MICRO-ORGANISMS

AND FERMENTATION

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BY

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FIFTH EDITION, RE-SET.

REVISED THROUGHOUT BY THE AUTHOR,

WITH THE ASSISTANCE OF

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PREFACE TO THE FIFTH EDITION.

THAT a fifth edition of this work has been called for notwithstanding the set-back of the Great War is most gratifying, and my Publishers with their customary thoroughness have undertaken the resetting of the work throughout so as to include the results of the large amount of important research which has been accomplished during the past decade, and for this I thank them, as it is scarcely possible that I shall be privileged to revise for any further edition.

To keep the book within reasonable compass, it has been necessary to condense the amount of recent progress, relating, for example, to the theories of fermentation and the description of the numerous new species of micro-organisms. In many cases it is not yet possible to put these in systematic order, because the specific characters given in the literature are not always exhaustive The descriptions I give will at any rate show the great multiplicity of races which is characteristic of this class of lower organisms

The scope of the book being more particularly to give an account of microbiological science in its bearings on the fermentation industries, in several of which an important evolution has been going on during these years, the chapters dealing with the *practical application* of scientific observations and facts had to be re-arranged and treated more extensively, this is more particularly true of these relating to the nutrition of yeast and the use of pure cultures in various industries.

After my manuscript was completed at the end of 1923, my friend, Mr S. H Davies, greatly obliged me by submitting the same to a thorough linguistic revision, for which I tender him my sincerest thanks.

ALFRED JORGENSEN.

COPENHAGEN, *January*, 1925

PREFACE TO THE FOURTH EDITION.

THE first edition of this work was issued in the form of a text-book in 1886 It was the first attempt to express the biological significance of the science of fermentation and of the fermentation industry, a field where the chemical point of view had hitherto prevailed

I was induced to give this form to my work by the fact that in 1881 I had established an institute in which my first aim was to treat the problems of the fermentation industries from a micro-biological point of view This necessitated a short course for technologists and chemists who wished to study the science of fermentation on new lines, and as both older and younger students were attracted to my laboratory, the subject-matter had to be arranged so that the book could serve as a guide, even to those who had no special preliminary knowledge During my co-operation with E C Hansen in that early period, the principles of the practical application of pure cultures were broadly outlined, and accordingly an explanation of the principles underlying the resulting technical reforms formed the essence of the treatise The new editions which appeared in rapid succession, showed that the attempt had been successful At the same time, I had the good fortune to introduce into breweries in many countries properly selected pure cultures of bottom yeasts, following the precedent of E C Hansen, who shortly before had introduced similar yeasts into the Carlsberg Brewery in Copenhagen At this early stage, I successively described pure cultures of yeast types which are used in other branches of the fermentation industry, and I introduced them into top-fermentation breweries, as well as into distilleries, yeast factories, fruit-wine factories, etc Thus a beginning was made in the application of this new principle to new fields of industry Consequently, to keep pace with these developments, together with the results of research in the science of fermentation, my book had to be so largely modified that each new edition became to some extent a new book, whilst the growing mass of material considerably increased the size of the book Having to keep within certain limits, I was reluctantly compelled to leave various problems

undiscussed On the other hand, repetition could not always be avoided, when similar subjects were discussed in different sections

Among the many new questions demanding fuller treatment in this, the fourth English edition, I may specially mention the fermentation of milk and other dairy products, the enzymes, and the conditions of yeast nutriment As regards the technical part of the work, a new section deals with methods of cleansing and disinfection in the fermentation industry, and the section dealing with the application of pure cultures to the various branches of the fermentation industry has been considerably enlarged In its present form the book will, I hope, serve as a manual, not only to zymo-technologists, but also to analysts and physiologists *

For convenience, the old names of yeast species have been retained along with the new, in the systematic treatment given in the fifth chapter

As the work is concerned with the micro-organisms of the fermentation *industry*, due regard has been paid to the practical application of research work, and the description of both useful and injurious species has received special attention The classification of the yeasts has been partially based on the same requirements The description of each species emphasises those characteristics that are of special importance to the industry.

I am glad to take the opportunity of expressing my thanks to the head of the students' department in my laboratory, Herr J. C Holm, the systematic part of Chap v, together with the very full bibliography, are essentially his work

Finally, I wish to express my warm thanks to many authors who have kindly sent me reprints of their published works I regret that the limited extent of my book has in many cases prevented me from making use of valuable publications

ALFRED JORGENSEN

COPENHAGEN, *January*, 1909

* A short description of the most important practical conditions found in the fermentation industry and in the laboratory is to be found in my short work, *Practical Management of Pure Yeast* London, 1903

CONTENTS.

CHAPTER I

Microscopical and Physiological Examination.

CHAPTER II

Biological Examination of Air and Water.

CHAPTER III

Bacteria.

CHAPTER IV

Moulds.

CHAPTER V

Yeasts.

CHAPTER VI

The Pure Culture of Yeast on a Large Scale.

MICRO-ORGANISMS AND FERMENTATION.

CHAPTER I.

MICROSCOPICAL AND PHYSIOLOGICAL EXAMINATION.

1 Microscopical Preparations, Staining, and Microchemical Examination.

The **Microscope** will always be the chief means for investigating micro-organisms, for these are, as individuals, almost always invisible to the naked eye.

The microscope is made up of a mechanical and an optical part The mechanical part, or **stand,** consists of the **foot,** the **stage,** the **tube** carrying the lenses, and the **adjustment** for regulating the distance between the lens and the object lying on the stage The adjustment is partly "coarse" (a screw engaging in a toothed gear attached to the tube) whereby the tube can be rapidly raised or lowered, partly "fine" (a finely cut screw) by means of which the tube can be gradually raised or lowered, after finding the object with the coarse adjustment The tube commonly consists of two telescoping parts A table, which usually accompanies the instrument and gives the scale of magnification, records the corresponding **length of tube** either in millimetres (usually 180 mm) or in inches (usually 10 inches)

The optical part consists of the **lenses** and **illuminating apparatus** (a mirror and Abbé condenser)

The lenses form the most important part of the microscope, the system turned towards the eye is called the eyepiece, and that turned towards the object is called the objective

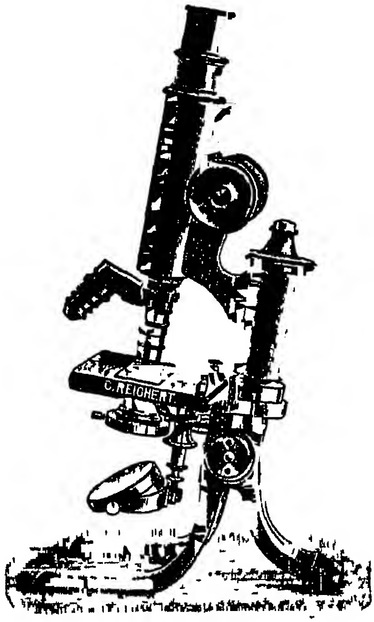


Fig. 1.

When a bundle of parallel rays of light strikes a convex lens, the rays are refracted and collect at a point on the other side of the lens, called the **focus.** The distance between this point and the lens is called the **focal distance.** If a small object is placed on the stage at a slight distance beyond the focal length of the lens and illuminated by the mirror, the rays passing through the lens of the objective will not be parallel, but will diverge, and so form a **magnified** image of the object The smaller the focal length, the greater the magnification This real inverted and magnified image formed by the

objective, which must fall exactly in the focus of the eyepiece, is seen through the latter as an imaginary, magnified image of the picture formed by the objective. The whole magnification secured by the microscope is, therefore, a product of the magnification due to the objective and that due to the eyepiece The magnification is always expressed as a linear and not as a quadratic dimension—i e, the relationship between the length of a line as seen through the microscope and its length as seen by the naked eye

From any given point of the object on the stage of the microscope a bundle of rays may pass through the lens The angle which the outermost rays of the bundle form is called the **angular aperture.** It is not customary to allow all the rays of the angular aperture to pass through the system of lenses which make up the objective. A larger or smaller number of the peripheral rays are excluded by means of diaphragms consisting of metal discs with round apertures, which are of various sizes, and fit into the opening on the stage In this way the actual angular aperture of the objective is reduced The peripheral rays would give an indistinct picture, but the picture would, on the other hand, lose in clearness if too many of the outer rays were excluded.

For this reason the objective is so constructed that the aperture shall be as great as possible An expression for the value of the latter is given by the numerical aperture (the sine of half the angular aperture multiplied by the index of refraction of the medium in front of the lens—air, water, oil, etc)

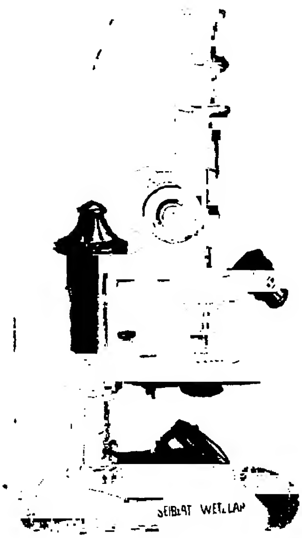


Fig. 2.

When the rays of light are refracted in the objective some of them are immediately split up into the component coloured rays, and the image shows coloured edges (**chromatic aberration**). To avoid this the objective is made up of several different lenses prepared from various kinds of glass (crown glass, flint glass) which possess different refractive powers It is thus possible to prevent any indistinctness of outline

To secure greater magnification a system of **immersion** is used, the space between the front lens of the objective, which is made of crown glass, and the cover-glass being filled with a strongly refractive medium, water or oil The immersion is **homogeneous** if, as is often the case, the oil has the same index of refraction as the cover-glass and the front lens. In this way an increase of the numerical aperture is secured, and, therefore, a greater **resolving power**—the limit of definition for the smallest perceptible details.

The **eyepiece** consists of two lenses, the upper, which comes into direct contact with the eye, and the lower, called the **collective lens,** which collects the rays of light so that the **field of vision** is reduced, and is, therefore, more easily surveyed. Between the two lenses a diaphragm is inserted in order to further reduce the field The collective lens is also of importance in securing, along with the upper lens, the exclusion of the coloured edges of the microscopical picture

The greater the curvature of the lenses in the eyepiece, the more do they magnify the image projected by the objective, which at the same time becomes darker and less distinct. To obtain a well-lighted field when working with high magnifications, it is necessary to use strong objectives and weak eyepieces

It is well known that the lenses of the human eye alter their shape according to the distance of the objects that are under observation They can accommodate themselves, and, by projecting, shorten the focal length, and thereby produce a sharp image on the retina On the other hand, by a reduction of the convexity the focal length is increased, and thus a clear image of distant objects may be thrown on to the retina If the eye is short-sighted or long-sighted, the tube of the microscope must be set to suit the focal length of the eye, and the size of the image will differ for abnormal eyes The normal focal distance is considered to be 250 mm, and **tables of magnification** are based on this. The actual magnification for any individual eye must be established by a special calculation with the help of a glass micrometer

The **illumination** of the object is secured by a mirror placed below the stage which is capable of movement in all directions so that the object can receive direct or oblique

illumination, and so that the mirror can be fixed at different distances from the object For low powers the plane side of the mirror is used, for higher powers the concave side. With ordinary magnifications it is of importance to secure suitable illumination, as the eye soon tires if the light is too strong Instead of the usual diaphragm in the stage, an **iris diaphragm** may be used, enabling the aperture to be reduced or enlarged by means of a number of sickle-shaped leaves sliding over each other To give illumination over a large surface (*e g*, in the examination of coloured substances), a combination of lenses known as a condenser is introduced between the mirror and the preparation When studying an object the separate parts of which can only be distinguished by differences in their refractivity, a narrow bundle of rays must be used, and this is secured by placing a diaphragm with a smaller opening in the aperture of the stage

The microscopical examination of the organisms of fermentation throws light upon their size, form, colour, the refractive indices of different parts of the cell, and, generally, of all those conditions which are the object of **morphological** research

As we are dealing with living forms, we can only arrive at a real knowledge of them by studying their life conditions, through biological and physiological research.

Biological research is concerned with the investigation of life phenomena under the conditions existing in nature; thus, such conditions as the distribution of single species, their occurrence, the numbers present in different localities at different seasons, their sensitiveness to light, to heat, to the moisture of the atmosphere, etc

Physiological research has for its object the study of the life history of the organism, the conditions of nourishment and propagation It is also concerned with the different kinds of fermentative activity, so far as these can be established by studying the influence of organisms on the liquids in which they are growing, and with the nature of the substances or forces causing fermentation (enzymes) Specially constructed apparatus is available for such investigations, and many of these lines of research are closely allied with chemical studies

One essential condition of any exact investigation into the life history of micro-organisms must be secured—the certainty that we are working with a single cell or with one vegetation, consisting of a single species, and, therefore, derived from one cell We shall see in the following pages how the technique has been slowly developed, and how this goal has been reached, as the result of many scientific and technical attempts to prepare absolutely **pure cultures.**

For the ordinary examination of yeasts and moulds, a clear magnification of 600 suffices For the examination of the fine details of these organisms and of bacteria, higher powers are required. Although an immersion lens is of great service, it is not essential for ordinary technical work

It is of real importance that the organisms of fermentation should be examined, as much as possible, in a **living** state, and either in a drop of water or of a liquid in which they have been growing. The drop is placed on an object glass, and spread out to

form a thin layer, by placing a cover glass on top of it, or else the drop may be placed inside a moist chamber (described later) in which the growth and propagation of the cells can be followed.

Certain characters, however, of the detailed construction of these organisms can only be detected by the use of special **drying** and **staining methods.** To do this, the cells are subjected to a thorough treatment with concentrated dyes, some of a poisonous character They are killed, and the possibility exists that the characters brought out by staining may differ somewhat from those of the living cell Drying may also modify the length and breadth of the bacteria On the other hand, the staining process has explained many phenomena which were not apparent by observation of the living cell

Dilute dyes (*e g*, eosin, methylene blue) are used in the technical examination of yeasts to obtain an idea of the proportion between dead and living cells in a vegetation, since the dead cells alone absorb the dye

As an example of a method of staining, we may instance the treatment to which **yeast cells** are subjected in order to observe the **cell-nucleus** and its subdivision Hoffmeister proceeds as follows —The young, vigorous yeast growth is washed several times with distilled water, and then before the actual staining it is subjected to the process of fixing, according to one of the recognised methods For instance, the yeast is stirred up with Bath's solution (consisting of a litre of a concentrated, aqueous solution of picric acid, together with 4 c c of glacial acetic acid and 1 gramme of osmic acid), the cells are thus coloured yellow. After allowing the mordant to react for 24 hours, the cells are again washed with water, spread out in a thin layer on a cover-glass, and allowed to dry. The preparation is then treated according to Heidenhain's method The cover-glass preparation is allowed to float in a Petri dish, on the surface of a solution containing 2·5 per cent of iron alum, after 6 to 24 hours the cover-glass is washed once with water, and then placed in a 0 5 per cent. aqueous hæmatoxylin solution After a further 24 hours the cells, as seen under the microscope, are stained deep black. They are then treated for a few minutes with a 0·25 per cent solution of iron alum, after which the yeast cells appear colourless with violet or greyish-black cell nuclei The preparation is mounted in undiluted glycerine. One of the more advanced of recent workers in this field, A Guilliermond, makes use of the method of staining just described to prove the presence of the cell-nucleus in the yeast cells; but for fixing he prefers picroformal (*Bouin*).

Rayman and Kruis prefer the following procedure :—Fixing with a solution of iodine in potassium iodide and alcohol, treatment of the cells with an ammoniacal iron-alum solution, coloration with alizarin PS (Bayer & Co, Elberfeld), and decoloration

with the same alum solution. The nucleus will thus be stained dark red in a colourless plasma

Wager likewise recommends a cautious treatment of the cells with Gram's solution of iodine in potassium iodide, or with a dilute solution of chromic acid, subsequently with alcohol, and drying on the object glass This method has the advantage of preserving the contents of the cell intact Wager made remarkable observations of the detailed structure of the nucleus by cutting sections through the cells with a microtome The coloured cells, after pouring off the colouring matter, were treated successively with methylated spirit, absolute alcohol, turpentine or xylol, and then fused into paraffin-wax.

To prove the presence of the fine hairs, which serve the bacteria as organs of movement, the **flagella** or **cilia,** which can seldom be detected by direct microscopical examination of the living bacteria, the following method (*Loffler*) is adopted —A small quantity of a very young growth of bacteria (developed for five to eight hours in an incubator) is placed in a drop of water—the ordinary supply is preferable to distilled water—and the contents of this drop are divided amongst a number of drops of water, placed on a series of carefully cleaned cover-glasses They are air-dried, and are then passed through a flame in order to fix the bacteria. Care must be taken that the preparation is not heated too strongly The simplest means of avoiding this is to hold the cover-glass between the fingers, and not to heat it more strongly than they can bear A large drop of a mordant is now spread over the heated cover-glass The mordant, which is applied to render the bacteria absorbent to the actual stain, consists of 10 c c of tannic acid solution (20 per cent.) mixed with 5 c c. of a cold saturated ferrous sulphate solution and 1 c c of a saturated aqueous or alcoholic fuchsine solution. The cover-glass is warmed for about half a minute until steam is given off, but violent boiling must be avoided The preparation is washed with a powerful stream of distilled water, and afterwards with absolute alcohol until the cover-glass is clear, and only the spot on which the water drop has been evaporated appears cloudy The **staining fluid** is now poured over the surface of the cover-glass It consists of a neutral saturated fuchsine solution in aniline. The preparation is warmed again for a minute until steam rises, washed with a stream of water, and is then ready for examination. It should be noted that all motile bacteria do not show their flagella when they are treated with a mordant of the above composition. One must proceed experimentally, for some bacteria require a mordant to which a few drops of a 10 per cent soda solution have been added, whilst others require an addition of sulphuric or acetic acid in place of soda. Loffler found that several of the acid-forming bacteria require an alkaline mordant, whereas a number of alkali-forming organisms require an acid mordant. By such exact means

beautiful pictures have been obtained, which show that bacteria are supplied with these organs of movement arranged in various ways, they often cover the whole surface of the cell. A similar treatment brings out clearly the coating of slime which, for instance, surrounds the cells of acetic acid bacteria, but is invisible in an ordinary microscopical examination.

In the method devised by Carsares-Gil, which has proved of value, he first prepares a solution by mixing up in a mortar 10 g. of tannic acid and 18 g. of moist aluminium chloride with 30 c.c. of alcohol (70 per cent). To this is afterwards added, drop by drop, a solution of 10 g. zinc chloride in 10 c.c water and 1·5 g. of rosaniline hydrochloride The solution thus obtained is kept in a dark place One part of this solution is mixed with four times as much water, allowed to stand for a minute, and filtered. The preparation, fixed on the object- or cover-glass, is covered with the solution for a minute and washed with plenty of water The flagella are thus stained, and the bacteria may be stained afterwards with methylene blue or carbol fuchsine.

Zikes (*Allgem Zeitschr. f Bierbr.*, Vienna, 1910) obtained favourable results by a combination of Löffler's mordant and van Ermengen's silvering process. As a mordant he uses 5 c.c. of a concentrated solution of ferrous sulphate (free from hydrate), which he adds, drop by drop, to a solution of 8 parts of tannic acid in 2 parts of water This liquid, at atmospheric temperature, is poured over the preparation in the course of one and a half minutes, then washed off, and the preparation immersed for a few seconds in a 0·25 to 0·5 per cent silver-nitrate solution, and subsequently for a few seconds in a solution of 5 g gallic acid, 3 g. tannic acid, 10 g. potass acetate, and 350 c c. water. The preparation is again transferred to the silver solution, and eventually to the tannic acid solution, until the bacterial layer begins to turn brown.

Another method often employed consists in fixing with a mixture of chromic, osmic, and acetic acids, and staining with ferro-hæmatoxylin.

The so-called **Gram-staining** is effected as follows:—After fixing the bacteria on the cover-glass, by cautiously drying over a flame, the preparation is treated with gentian violet, methylene violet or Victoria blue for one to three minutes, and after washing with water, a solution of iodine in potass. iodide is added and allowed to act for two minutes, without warming. After pouring off the solution, the residue is washed with absolute alcohol, until no more colouring matter is removed The preparation should then be washed with water, dried, and mounted in Canada balsam. The bacteria that are stained are called Gram-positive, the others Gram-negative According to Lehmann and Neumann, and also to Löhnis, this reaction is not, however, always reliable, as one and the same species may react differently according to its age or

previous treatment, even individuals in one and the same pure culture were sometimes found to react differently.

Neisser's **granule staining** requires one of the following:—

(a)	Methylene blue,	1
	Alcohol (96 per cent),	20
	Aq dist,	1,000
	Glacial acetic acid,	50
(b)	Crystal violet, not exceeding	1
	Alcohol,	10
	Aq dist,	300

In a few cases staining has proved of value in determining the species, this is the case with the acetic acid bacteria investigated by E C Hansen, *Bacterium aceti*, *B Pasteurianum*, and *B Kutzingianum* Staining is most readily done by treating a vigorous growth with an aqueous solution of iodine in potass iodide, or an alcoholic solution of iodine The slime surrounding the cells of *B aceti* is coloured yellow, whereas that of the other two species is coloured blue The latter reaction is brought out more clearly when the slime is forced out sideways by pressure on the cover-glass Blue coloured flecks are thus formed, while the cells themselves are either colourless or yellow.

Photographs of micro-organisms are now frequently employed. The preparation may be first stained, for instance, by Löffler's method, to bring out the characteristics more sharply, and to remove foreign bodies Whilst the usual sketches of microscopical preparations are always more or less diagrammatic, omitting everything except the special characters it is desired to emphasise, micro-photography provides a more correct representation of the object, and has a further incidental advantage It is well known that the photographic plate is sensitive to certain chemically active rays of light which cannot be detected by the human eye, so that photography can reproduce certain characters of the preparation which would be entirely missed by direct observation. It may be anticipated that the new method of photographing by ultra-violet light will bring about notable advances, for it will thus be possible to distinguish a greater number of fine characteristics and preparations otherwise colourless will appear to be composed of differently coloured parts.

The **ultra-microscope** makes it possible to distinguish small details that are invisible with all earlier optical devices Its design is based upon a special method of lighting, discovered by Siedentopf and Zsigmondy, whereby the preparation is brightly illuminated with rays of light, falling in a direction at right angles to the axis of the microscope The illumination is so arranged that a single layer of the preparation is lit up whilst the remainder is in shadow, With the help of this apparatus particles can be distinguished

of a size of four-millionths of a millimetre (The symbol μ is used for $\frac{1}{1,000}$ mm and $\mu\mu$ for $\frac{1}{1,000,000}$ mm) The effect of such an illumination may be realised by recalling the well-known appearance of particles of dust floating in a beam of sunlight penetrating into a darkened room, when the particles are viewed in a direction approximately at right angles to the beam of light

A **micro-chemical** examination is of value for ascertaining the composition of the individual parts of a micro-organism After treatment of the cells with reagents, the reaction brought about, and especially the colour reaction, may be studied under the microscope, with a view of determining the chemical composition of the part in question. As an example the proof may be cited that yeast cells, in the later stages of development, contain the reserve food stuff, **glycogen** (a carbohydrate) In order to detect this substance in the cell an iodine solution is added to the preparation (2 grams iodine, 6 grams potassium iodide, and 120 c c of water) The albuminoid portion of the cell contents is thereby coloured bright yellow, whilst the glycogen assumes a reddish-brown colour. A further proof that the coloured part of the cell contents really consists of glycogen is obtained by warming the preparation to 70° C , whereby the colour reaction of the glycogen disappears , on cooling down the colour reappears If the cells are cautiously pressed under the cover-glass, so that they burst, it may be observed that the brown coloured glycogen is liberated as a fluid mass, which quickly dissolves in the surrounding liquid

Oil or fat globules are often found in the fully developed yeast cells. By the addition of 1 per cent osmic acid they are coloured brown ; sometimes they assume a vermilion colour with alcanna tincture, and frequently they may be dissolved by treatment with alcohol-ether benzene, or carbon bisulphide The treatment must, however, be continued for some time , the solution takes place more rapidly if the cells are crushed and the solvent brought into direct contact with the globules of oil The **albuminoids** of the cells, as already stated, are coloured yellow, or yellowish-brown, with iodine , with nitric acid and ammonia they are coloured lemon-yellow, and with Millon's reagent (mercuric nitrate), brick-red.

In the examination of fermented liquids micro-chemical methods are used to determine the character of any suspended matter other than micro-organisms To recognise clearly the reaction of the tiny particles floating in the liquid, it is sometimes necessary to separate them by centrifugal action, and further to wash them free from the liquid. The **starch** or **paste cloudiness** which occurs in beer is produced by fine particles of starch or dextrin, which separate out when the liquor contains a given quantity of alcohol They may be recognised by the addition of iodine, whereby they are

coloured blue or brownish-red (dextrin) A **cloudiness** due to **albuminoids** is often produced, it is due to flocculent, membranous, and often very irregular agglomerates, and to minute granules, which are either isolated or enclosed in these secretions. Such a formation frequently occurs in pasteurised beer, it may be distinguished under the microscope by the readiness with which it absorbs the colouring matter from iodine or from aniline dyes, it is coloured brick-red with Millon's reagent, and rose-red with Raspail's reagent (sugar and sulphuric acid) These bodies may also acquire a yellow colour (through the formation of xanthoproteic acid) by treatment with nitric acid and then with ammonia, or sometimes with the acid alone **Glutin cloudiness** is a frequent form of albuminoid precipitation, it takes the form of a fine veil throughout the liquid At 30° to 40° C the liquid becomes clear, on cooling, it again becomes cloudy Under the microscope, fine flecks and small yellowish granules can be seen. According to Will, the latter consist of sheaths, more durable than the contents, which are easily attacked by water or dilute alcohol, acetic acid, or hydrochloric acid, and thus the sheaths become recognisable, in 5 per cent. potash, the whole granule dissolves. On warming, the contents, but not the sheath, are dissolved; hence the warm liquid is not always absolutely clear Occasionally a **cloudiness** due to **hop resin** occurs in beer, the yellowish-brown globules are recognised by the vermilion coloration given by an alcoholic alcanna solution A **cloudiness** of beer due to a **resin** derived from pitch has been described by Will, precipitations of this kind are coloured violet by a mixture of acetic anhydride and concentrated sulphuric acid This reaction is specially marked when the granules are separated from the liquid.

2. Biological Research by means of the Microscope; Moist Chambers.

The examination described in the previous section can give but a limited insight into the nature of micro-organisms A more complete knowledge of their life characters can only be attained through a biological and physiological investigation The methods adopted have gradually reached a high stage of development, and **micro-biology** now stands as an independent branch of natural science, which has given results valuable both to science and to industry.

The subject of micro-biological research may be either a **growth** or an **individual, a single cell.** In the first case, the certainty of the result is determined by the purity of the growth, and whilst the work is in progress this must be secured by the adoption of special precautions to be further described In the second case, with which we now have to deal, the entire examination must be

carried out under the microscope, special means being required to enable us to observe the series of changes that arise from the development and growth of the single cell With this aim in view, Ranvier's moist chamber may be used (Fig. 3) This apparatus

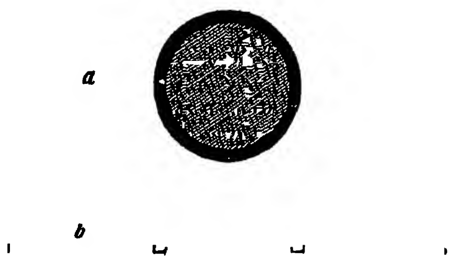


Fig 3

is made by grinding a slight hollow in the middle of a common object-glass; round this hollow a groove is made of greater depth to carry water The drop of nutritive solution, which must be very small, is placed in the middle of the hollow and covered with a cover-glass, which extends beyond the groove. When the cover-glass is in place, it is cemented by means of vaseline, and the drop is thus enclosed between the cover-glass and the hollow of the object-glass, whilst the water in the groove prevents evaporation.

If by suitable dilution, care has been taken that only one cell has been sown in the drop of water, the study of its development may be extended for any length of time, with the certainty that all forms that appear are derived from one and the same individual. It is obviously a condition of this and all similar investigations that the liquid and the closed part of the apparatus must be sterile.

This chamber may be used again to decide whether fine particles floating in a liquid are secretions or bacteria Substances are added to the liquid which favour the growth of bacteria, and by prolonged observation of the behaviour of the particles it may be determined whether they propagate or not

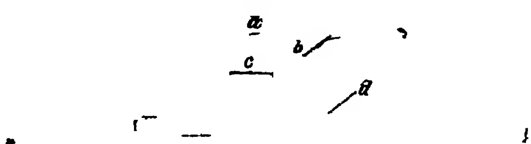


Fig 4

Amongst various kinds of moist chambers, that of Bottcher (Fig 4), which now finds extensive use, may be mentioned It consists of a glass ring fastened to a common object glass, and upon this a cover-glass is cemented with vaseline The cover-glass

carries on its under side a freely suspended drop containing the object to be examined A few drops of water are placed on the floor of the chamber to prevent evaporation of the suspended drop. If the cover-glass has been completely freed from grease by cleaning with acid and ether, the drop will spread out to a thin layer, so that it may be observed under a strong power, and with a short focal distance. As the drop hangs freely, it is possible to lift the cover-glass without disturbing the growth, if a sample is to be removed If the cells are to be fixed, a little gelatine may be mixed with the liquid before sterilisation, as suggested by Brefeld. In his detailed researches on micro-organisms, Brefeld used this and other kinds of moist chambers, which are illustrated and described in Vol. IV of his *Botanische Untersuchungen uber Schimmelpilze* (Leipsic, 1881). In order to secure the presence of a single cell in the drop, he diluted the infected liquid until this proved to be the case If the organism demands a full supply of air to reach complete development, a stream of moist air may be passed through two tubes fixed in the sides of the glass ring

By the help of such apparatus definite conclusions can be arrived at regarding the nature of the growth of micro-organisms, and this knowledge is essential for accurate work with mass cultures. To prepare the latter, the small pure culture is transferred with every precaution from the moist chamber to a flask containing sterile liquid (see detailed description in a later section).

3 Sterilisation.

The principles of the whole technology of sterilisation, as well as the different kinds of apparatus required, were described in the early memoirs on spontaneous generation.

The details of the development of this subject in its historical setting are given with the description of the theories of fermentation in Chap. v

Sterilisation of objects, whether a liquid or a piece of apparatus, means the riddance therefrom of all germs capable of development. This may be carried out either by removing all germs by mechanical means or by killing them by heat, or by the use of antiseptics. The choice of treatment is determined by the composition of the object to be sterilised, and obviously those means will be chosen that will render the adhering germs harmless, whilst producing the minimum of change in other directions Sometimes, with this in view, the sterilisation can only be partial, for instance, if the properties of the liquid are changed to a great extent, by heating to the temperature at which the germs present would be killed, the lowest temperature must be found by experiment at which the organisms are so greatly enfeebled that they are no longer

able either to develop or to affect the liquid This is the object of pasteurisation, which will be discussed later on

One circumstance which often presents great difficulties to complete sterilisation is this—that the great majority of micro-organisms occur in two different forms of growth, vegetative cells and spores. Whilst the former are usually killed at a temperature below the boiling point, the latter, and especially the spores of bacteria, can withstand prolonged heating at the boiling point, thus Flugge has isolated a species of bacterium from milk, the spores of which withstood boiling for four hours. When such spores are encountered, it is necessary either to boil for several hours, or to adopt a considerably higher temperature, the latter alternative is specially necessary when dry heat is used.

(*a*) **Sterilisation of Glass and Metal Objects.**—Sterilisation must always be preceded by a thorough mechanical, and often by a chemical, cleansing. Articles of daily use in the laboratory, such as spatulas, needles, wires, etc, are heated directly in a flame, and allowed to cool in a space free from germs. Many pieces of apparatus, however, do not admit of this treatment, harm may be done by overheating while ensuring that every part of the object has been sufficiently heated, or the number of objects to be sterilised may be so great that it would take too much time to treat each singly The apparatus must in this case be placed in special **sterilising ovens,** where it is exposed for some time to a temperature at which it is believed that all germs will be destroyed.

Dry or **moist** heat may be used according to the nature of the article. Dry heat is a much weaker disinfectant than moist heat at the same temperature To make certain that all germs are killed when using a hot air steriliser, the air must be raised to a temperature of 150° to 160° C, and the articles must be subjected to this heat for one to two hours. Some objects are wrapped in paper, others (*e g*, flasks) are closed with a cotton-wool plug, which should be covered over with filter paper. If moist heat is required, the object can either be boiled in a water bath, or, better still, subjected to the action of steam It is obviously of importance to see that the air is completely driven out, so that it cannot form a protecting layer, and prevent the steam from coming into contact with the object

Either a current of steam may be used, or steam under pressure In the first case, the apparatus is placed in a vessel provided with a perforated false bottom, with a sufficient quantity of vigorously boiling water below it The steam escapes slowly, as the lid of the vessel is not air-tight, and the apparatus is gradually raised to the boiling point By boiling in steam at 100° C all vegetative forms are probably killed, together with many spores of bacteria and other resistant forms if the treatment is continued for an hour.

But in many cases it will be necessary to employ higher temperatures, and it has been shown that the disinfecting power of steam rapidly increases when its temperature rises above 100° C Steam under pressure is, therefore, used in a **Papin's digester** or **autoclave,** constructed to stand a pressure of several atmospheres This apparatus is specially useful for the sterilisation of several liquids used in the laboratory If small quantities of liquids are to be sterilised, a pressure of one atmosphere, corresponding with a temperature of 120° C, is sufficient, if applied for half an hour. During the cooling of any variety of sterilising apparatus, care must be taken that the incoming air is sterile, and this is secured by passing it through sterilised cotton wool

(*b*) **Sterilisation of Liquids and Solid Nutritive Substrata.**—All germs can be removed from nutritive liquids by suitable **filtration;** but this method of treatment, which is more troublesome than heating, is only used for liquids when their composition is affected by heat Even in this respect it must be noted that filtration is not without effect, for the investigations of Flugge, Arloing, and others have shown that a filter retains or reacts upon certain of the soluble constituents, for instance, upon certain enzymes As the filtering medium, either burnt clay, plastic charcoal, gypsum, asbestos, or kieselguhr may be used The pores of these substances are very fine, and a thick layer must be used to ensure that even the smallest bacteria are retained The pores are soon stopped up, and the filtration must then be hastened by pressure or suction In laboratories, and for filtration of small quantities of water, the Pasteur-Chamberland filter, consisting of burnt porcelain clay, and Berkefeld's filter made of compressed diatomaceous earth are frequently employed These filters take the form of a hollow candle, closed at one end, the liquid flows into the hollow and out by a tube fixed at the other end of the capsule To test such a filter, it is immersed in water, and air is blown into the cavity If bubbles of air rise through the water, the filter is evidently perforated, and is, therefore, useless The first runnings of a filter, even a perfect filter, are not always sterile, and after a filter has been in use for a short time germs always pass through it This happens because the germs at length grow through the pores, since it is almost unavoidable that substances which supply nutriment to the bacteria should penetrate into the filtering medium The surface of the filter must, therefore, be frequently cleaned, and the filter sterilised, which is most easily done by boiling it in water

Of late years parchment-like **Membrane-filters** have come into vogue, formed by evaporating solutions of certain colloids under definite conditions The width of their pores may vary within wide limits, in the most effective of these filters the width is 1 μ or less This kind of filter is employed both in chemical and biological research work, for filtering wine, beer, etc

In breweries, the filtration of beer has been resorted to for some years, the filtering media commonly used being paper, cellulose, asbestos, etc. By such filtration brewers sometimes succeed, it is true, in freeing a beer of sound origin from deposits of various kinds, and in rendering it bright, but, on the other hand, it has been directly proved by Thausing, Wichmann, Reinke, Lafar, and others that an indiscriminate employment of this method may prove extremely dangerous. If the filters are not effective, it may happen that only yeast cells are retained and not bacteria, which can then react with much greater energy upon the liquid. Another great danger lies in the fact that a filter, when it is imperfectly cleansed, may harbour colonies of different kinds of germs, causing the contamination of all beer passing through it If a single cask in a cellar has become infected, and the filter is not effectually sterilised after the filtration of its contents, the disease will be communicated to the whole of the beer. It is, of course, a great mistake to use a filter which has been allowed to stand for a day without previous sterilisation, the different species will have rapidly propagated in the favourable substratum, and will be swept off by the following filtration At this stage of development the cells of wild yeasts are much more vigorous than those of the cultured yeasts, so that the disease organisms will multiply rapidly, and cause serious infection A warning must be given against treating the filter with water at a temperature below the boiling point, a thorough cleansing can only take place by prolonged boiling By careful handling of the excellent filters now manufactured by several makers a more stable product can be obtained than that before filtration, as the experience of the author has shown It is certainly not allowable to lay it down as a general rule that beer must always be spoilt by filtration

The filtration of **milk** of any biological importance has proved, so far, impossible, as a filter with pores large enough to allow the fat globules to pass will not retain bacteria, of which the vast majority are smaller than the globules. The filter is, therefore, only of use in removing the greater part of the dirt particles from the milk, and the micro-organisms that are attached to them On a large scale sand and gravel filters are used. For instance, in the Danish system, as constructed by Busck, a vertical cylinder is used with perforated diaphragms, between which are packed layers of sand, the grains of which are coarser at the bottom and finer at the top. The milk is run in from below. In Kröhnke's construction the milk is passed through a cylinder partially filled with gravel, and carrying vertical diaphragms, the cylinder is rotated round a horizontal axis. On a small scale, the fresh warm milk may be filtered through cotton wool, a layer of which is inserted between two sieves, the filter requires renewing daily. A

more complete biological purification has been attempted by pasteurisation

The filtration of **air** is intended, not only to remove living germs, but also to remove all floating particles Schroder and Dusch accomplished this by means of a tube filled with closely packed cotton wool, and this still proves to be one of the best materials In the laboratory such filters are used to seal test tubes and flasks When they take the form of glass tubes, as in the Freudenreich flasks, it is unnecessary to protect the small surface exposed at the open end of the tube, but it is otherwise in the case of test tubes, where a great part of the filter is exposed to the dust of the air Germs may easily grow on the cotton wool when it absorbs moisture Flaming the surface is not always sufficient, and in such a case it is desirable to keep the tubes in an atmosphere free from germs. By the diffusion of air, which goes on through such small filters, evaporation takes place, and as a consequence the liquid becomes more concentrated, or the gelatine hardens on the surface Such evaporation can be avoided by the use of the flask constructed by the author (Figs 8 and 9) On a large scale in breweries, yeast factories, etc, cotton wool packed in suitable vessels is also used for filtering air, or else the air is led through a large number of layers of cotton wadding (Moller's filter). The complete sterilisation of the air on the large scale cannot always be attempted, and could not always be justified from an economic standpoint.

In the filtration of **water** on the large scale, the conditions existing in nature are imitated, where water is allowed to sink through successive layers of soil, and the organic residues and micro-organisms are deposited on the finer layers, until, at a given depth, the water is sterile Artificial filters constructed of a number of layers of varying coarseness were first applied in London, and are now used in every country. Such a filter consists of a bed of large stones, covered with several layers of flints successively reduced in size so that the topmost layer is about the size of a pea, and on this is laid a layer of sharp sand about 5 feet in thickness, which has previously been washed The water is first stored in a reservoir, where the larger particles settle out When the filter is used for the first time water is led in slowly from below, so that all the air is driven out of the filter It is then allowed to stand quietly for some hours before the true filtration begins This must be carried out slowly at first, and then more rapidly It has been shown that the distance between the separate particles of sand is greater than the bacteria, and, therefore, the retention of the bacteria is not due to the sand filter. While the water is standing quietly over the filter, slimy matter in suspension settles down and forms a fine skin of slime on its surface This retains a few bacteria, and as it always contains organic residues, it supplies

nutriment for the bacteria, and as a consequence they multiply. A few bacteria settle on the grains of sand in the upper part of the layer, and these become slimy, and so arrest the bacteria subsequently carried down with the current of water In this way the upper part of the layer of sand gradually fills up, so that the pores between the slimy grains of sand are now smaller than the bacteria, and then, for the first time, it can act as a true filter. It is now "ripe," and the water in the lower layers of sand will be found to contain few bacteria. The necessary condition for satisfactory working is that the water shall flow slowly, in order that the bacteria and other particles may have time to settle on the slimy grains of sand, and also to prevent the skin breaking or the formation of channels through the bed of sand. The rate of flow must depend on the nature of the supply. If rich in bacteria, it should not sink more rapidly than 2 to 3 inches per hour For the same reason the water level must be retained within certain limits. During the slow passage of water, the bacteria embedded in the upper part of the sand are able to retain some of the dissolved organic matter in the water, so that when it leaves the filter it should be free from fermentative and putrefactive components. A stage is reached at last when the pores are so completely filled with bacteria that the capacity of the filter is greatly reduced, and it is then renewed by removing the top inch of sand. This process can be repeated until a layer of sand 16 to 20 inches thick is left, the layer must then be restored to its original thickness.

The important part played by biological conditions in the sand filtration of water has been clearly proved by the very thorough-going investigations of Kisskalt, who established the fact that *Protozoa* (*Flagellata*, *Ciliata*) are particularly active in checking *bacterial* growth Throughout the filter-bed they are indeed attacked and eaten up by such Infusoria, which occur generally in all water supplies In the water surmounting the filter, the bacteria are the first to multiply, followed by the Protozoa, which accordingly bring about a preliminary, but often a fairly effective, purification of the water, even before it has passed through the layer of mud This action of protozoa is borne out by the observation that the addition of 0 01 per cent. potassium cyanide, which kills the protozoa but not the bacteria, causes the latter to grow exuberantly throughout the whole filter and filtered water, whether the mud layer remains undisturbed or not The well-known fact that bacterial filtering capacity is diminished by low temperature, is considered by Kisskalt to be a purely biological phenomenon, the multiplying power of the protozoa being impaired by the lowering of the temperature, the way thus being paved for the growth of the bacteria. His researches establish the fact that an essential part of the purification process takes place within the sand layer itself, and he obtained favourable results even without a filter film and without a layer

floating on top of the sand. The sand layer must, therefore, not be too shallow. The filtering effect is largely influenced by the number of germs; it has been established that, given a very large number of germs, few are kept back, compared with those retained when they are less numerous. To sum up, the purifying effect—apart from the duration of the initial operations—is dependent on temperature, and also on the number and specific differences of the germs contained in the water.

Considerable use is also made of "rapid" filters. They may be constructed of sand, wood charcoal, etc., so as to allow of the passage of large quantities of water. Filtration is combined with chemical precipitation, whereby the greater part of the suspended particles and organisms is retained.

Ultraviolet light possesses high germicidal power, a direct effect of light rays of short wave-length on bacteria and living plasma. A particularly powerful effect is produced by the use of a mercury-vapour lamp of pure quartz. To obtain the effect, the water must be freed from all colloidal matter apt to cause turbidity, the brightness of the water being of some consequence. If it is rich in germs, the effect will be diminished. The different species appear to have different powers of resistance; thus, according to Oker-Blom, the water bacteria are less resistant than the coli species and several others. He states that bright colourless water containing about 10,000 germs per c.c. could be sterilised when the rate of flow was 50 to 90 litres per hour. According to Moufang, considerably greater effects are obtained with lamps of the most recent construction. The chemical composition of the water appears to be unaltered.

The treatment of water with ozone is dealt with in a subsequent section.

The exact method of sterilisation of liquid and solid substrata by means of **heat** is determined by their chemical and biological nature. The methods employed include the use of a current of steam, steam under pressure, boiling in water or on the sand bath, and the treatment may be prolonged for a considerable period if it is desired to kill not only vegetative cells, but also spores. In either case it is obviously of importance to take care that during the subsequent cooling only sterile air is admitted to the vessel. This is secured in the case of Pasteur flasks by the use of a tube with two bends, in which any germs that are sucked in are deposited; in the case of Erlenmeyer and Freudenreich flasks, by sealing them with cotton-wool filters. Whilst the hopped wort commonly used in zymophysiological laboratories will stand boiling on the sand bath, and after a comparatively short boiling can be preserved unchanged, wort gelatine and other gelatines cannot stand treatment on a sand bath or such prolonged boiling on a water bath or in steam, that will ensure the destruction of all spores, because

there is always a danger that after such treatment the gelatine will no longer solidify at a temperature of 25° C. The same difficulty is met with in the sterilisation of the mash in distilleries and of wort in the air yeast factories, owing to the great separation of albuminoid substances which takes place at the boiling point, causing a complete change in the character of the liquid; for this reason it is impossible to apply all the results of experiments obtained with properly sterilised liquids of this kind to the very different circumstances that obtain in practice In all such cases use is made of the method of *fractional or discontinuous sterilisation* introduced by Tyndall Its object is to bring about the germination of spores of bacteria and similar resistant organisms by maintaining the material at a gentle heat for some time, so that the cells may subsequently be killed at comparatively low temperatures. The material is first warmed, perhaps to a temperature of 70° C., or it may be heated for a quarter of an hour in a current of steam in order to kill the vegetative cells It is then maintained at room temperature, or, better still, at the most favourable temperature for the development of spores (about 35° C), and after the lapse of a day, or even of a shorter period, when it is assumed that germination is complete, the material is again heated. By repeated treatment of this kind it is possible to eliminate all spores and to kill all vegetative cells This obviously depends, however, upon the regular germination of the spores The treatment does not absolutely guarantee sterility, and before either liquids or gelatines are used they must be kept under observation for a considerable time. In many cases filtration is to be preferred to discontinuous sterilisation The liquids in daily use that are prepared with the help of micro-organisms, beer, wine, vinegar, etc., always contain a residue of these micro-organisms in a more or less vigorous condition. It is desirable, by heating them, to arrest the fermentation. The safest course is to sterilise the liquids, but as the temperatures required to effect sterilisation usually produce great changes in the liquids, it is necessary to limit the treatment to a temperature that will suffice to weaken the micro-organisms, so that under normal conditions they are extremely unlikely to propagate or to bring about further fermentation ("*pasteurisation*"). It is difficult to determine the best method when the nature of the liquid will not admit of a high temperature being used, while the result must depend upon the character and the activity of the different micro-organisms present, as well as upon the chemical composition of the liquid It is, therefore, impossible to establish any general rule It is essential in each case to determine experimentally both the temperature and the time of treatment, after forming a judgment as to what micro-organisms are present in the liquid. In the case of beer different temperatures are used—heating from 50° to 60° C. for two hours, or from 65° to 70° C. for

half an hour or more—and for wine, two hours' heating at 45° to 50° C. (*C Schulze*). A slow cooling down after pasteurisation has often been proved experimentally to give better results than rapid cooling. The determination of the right temperature is obviously rendered more difficult if the liquid harbours different species of yeast, and still more so if at the same time the development of bacteria has taken place, especially those species that form spores. It has been proved that when the heating exceeds certain limits, the flavour either of beer or wine quickly deteriorates, which is probably due in the first place to the decomposition of albuminoids.

If the liquid is particularly sensitive to high temperatures, it is necessary to fall back on the method of discontinuous treatment, whereby the liquid is heated to a moderate degree several times, with a suitable interval between each heating. Frequently the alteration in taste produced by pasteurisation can be partially removed by subjecting the liquid for a time to a low temperature. A special difficulty met with, particularly in the case of beer, is that during storage or transport, particularly at low temperatures, the pasteurised liquid develops a turbidity, or forms a deposit, consisting usually of albuminoid substances separating in the form of small granules, or, in difficult cases, in flakes and skin formation It has usually proved necessary to control the preparation of the malt if such a calamity is to be avoided Care must be taken that a slow and sufficiently advanced development of the grain has taken place, accompanied by a full transformation of its contents Further, it is obvious that the fermentation should have been vigorously carried out, and in this connection it is particularly necessary to adopt pure ferments. By cooling the beer to a low temperature before filtration and pasteurisation it is possible to avoid the subsequent separation, as part of the material in question is separated in the cooling process.

As regards pasteurisation of **vinegar,** it appears from Henneberg's experiments that the acetic bacteria will die off at lower temperatures in high percentage vinegars than in vinegar of low concentration. If the vinegar is heated to 48°-50° C, or kept two minutes at 46° C, it will be entirely freed at any rate from living acetic bacteria, warming to 40° C for twenty minutes will usually have the same effect. The dreaded vinegar-eels are killed at the same temperatures, they seem to be somewhat more resistant than the bacteria It goes without saying that when slime-forming bacteria are growing freely in the vinegar, the action must be somewhat more vigorous, as the bacteria are protected by the slime coating.

In dealing with **milk,** heat is applied in the same way. In this case the greatest possible difficulties are met with owing to the great range of micro-organisms present in milk (lactic acid bacteria, putrefactive bacteria, hay and potato bacilli, etc), many of which

are only killed at a high temperature, owing to their power of forming spores. Heating the milk further results in separating or modifying components, which may be of extreme value in nutrition (*e g*, the enzymes), even at comparatively low temperatures. Pasteurisation at temperatures considerably below the boiling point may result in the milk bacteria being killed, whilst the putrefactive bacteria remain alive As a consequence, the latter, freed from competition, multiply rapidly, and form putrefying matter in the milk, and this may occur to a considerable extent if the milk is not stored at a very low temperature. Actual sterilisation can only be secured if the milk is heated for an hour or more at a pressure of half an atmosphere, corresponding to 112° C If the object is simply to destroy the pathogenic organisms that are present, especially the tubercule bacilli, it is only necessary, according to Bang and Weigmann, to heat for a few minutes at 85° C, or for a quarter of an hour to an hour at 65° C. The problem how to secure a product free from any organisms capable of development, and yet of full nutritive value, has not yet been solved.

O Jensen recommends heating for half an hour at 63° C. and then cooling down to below 14°, or, better still, below 10° C, a treatment which several lactic acid bacteria will survive in ordinary milk Prolonged heating at the above temperature he believes to be dangerous, because the development of thermophilous putrefactive bacteria will be thereby facilitated

A peculiar form of pasteurisation of milk is the "**Biorisation**" devised by Lobeck, which consists in suddenly heating the milk up to 75° C by spraying it in extremely small drops through a nozzle, under a pressure of 4 atmospheres, into a space where this temperature is constantly maintained, and, after a few seconds, cooling it as suddenly in a Liebig's condenser. According to Schmitz, milk thus treated completely retains the nature of raw milk, as regards taste, smell, ferment reactions, and bactericidal power; only the faculty of coagulating by rennet was almost imperceptibly impaired. The common milk germs, apart from those forming spores, were destroyed, and so were the various disease germs, including the tubercle bacilli As, however, bacterial spores are not killed, the milk, when left to stand, may be decomposed by the bacteria developed To prevent any ill effects of their action—*e g*, the peptonisation of albuminoids—lactic acid bacteria may be added after biorisation

A Muller's researches proved that if the catalase of milk is destroyed by warming up to 70° C, and the milk subsequently cooled down, while any after-infection is obviated as far as possible, the addition of 0·1 to 0·15 pro mille of H_2O_2 will suffice to keep the milk fresh for three to seven days, even at summer temperatures, without any appreciable alteration in taste The same milk, if not submitted to such treatment, regularly deteriorated in 24 to 48 hours.

Milk treated and exposed to infection from the air while cooling kept fresh at least 24 hours longer than untreated milk

Sterilisation of **air** can be best secured, as already stated, by means of cotton wool filters Sulphuric acid or brine baths, cloth filters, etc , are less effective. In the laboratory, where it is often necessary to carry out work in sterile air, glass cupboards are used, the front of which can be sufficiently raised to admit the arms Some time before using the cupboard the whole of the inner surface is washed over with either mercuric chloride solution or 60 per cent. alcohol, and the cupboard is then closed Any particles and germs floating in the air will sink to the moist floor, and will be retained there.

In breweries and other branches of the fermentation industry, the fermentable liquid is sterile at a particular stage in the manufacture, at the moment when the boiling is completed. After the zymotechnical analysis of air had shown that it may convey disease germs to the fermenting liquid, attempts were made to protect the wort during the cooling operation against such infection by the use of closed cooling and aerating apparatus, closed fermenting vats, and storage casks, and by the sterilisation of the incoming air through cotton-wool filters These precautions, together with the use of an absolutely pure yeast, should, theoretically, produce an absolutely pure product Incidentally, one important practical object was secured, for by blowing in a powerful stream of air during the fermentation, and by the removal of carbon dioxide, the rate of fermenting was greatly increased, and an earlier clearing of the liquor took place The difficult problem is to maintain such large vessels in a condition of absolute cleanliness

The experience of many years has, however, shown that in breweries with open refrigerators and cooling apparatus, open fermenting vats, and ordinary storage casks, a product can be obtained with such a small content of harmful germs that they have no practical influence on its quality, notwithstanding the fact that the wort, especially on the refrigerators, is exposed to a number of foreign germs It has now been proved that the harmfulness of the atmospheric germs in the fermentation industry has been greatly exaggerated, for in competition with the enormous number of yeast cells which are established in the wort, the vast majority of these germs never come to development. If it happens that, notwithstanding the use of pure yeast, the product is strongly contaminated with disease organisms, the explanation is, in the great majority of cases, that these are developed in the plant itself It is from the surfaces of the different vessels employed that the dangerous carriers of disease have developed, just because a rational method of cleansing has not been adopted The chief importance must be attached to those stages in the process where the liquid is longest under treatment, in the fermenting vats and storage casks In order to purify these vessels, as well as the

connections, disinfectants are almost always used, and it may be remarked that a summary treatment with these is not sufficient. This, at any rate, holds good for wooden vats, in which it has often been proved that notwithstanding disinfection the disease germs retain their hold. A special investigation must, therefore, be made into the physical character of the vessels, and the necessary precautions must be adopted. If in this way a rational method is worked out, it will be found that the atmospheric germs exercise no noticeable influence on the course of fermentation or on the character of the product, since no opportunity is given them to establish themselves in the plant

Under special circumstances chemical reagents are used for disinfection, the **antiseptics.** The ground work of the technical application of antiseptics was laid by Schwann, who proved in 1839 that yeast cells die under the influence of certain chemicals. Subsequently the knowledge of antiseptics was greatly extended by R Koch As in the case of the action of heat, so the individual species react differently towards the various antiseptics. Moreover, one and the same species of vegetation may react differently towards the same reagent, and that not only because the spores possess a greater power of resistance than the vegetative cells, but also because the condition of the latter plays a part One practical problem is to determine how far the antiseptic can be diluted without ceasing to react. Whilst with a given concentration the antiseptic may prohibit life, with a greater dilution the action only restricts development, and with still greater dilution, if any further influence is felt, it may take the form of stimulating both the development and activity of the organism Many organisms possess a special power of accommodating themselves to strong doses of antiseptics.

Disinfectants are placed on the market either in a solid or in a concentrated liquid condition Their antiseptic power must first be determined by experimenting with the groups of micro-organisms which may be encountered in the fermentation industry Once the limit of their activity is determined, it is necessary to ascertain how rapidly a given dose operates Should it prove that the action is too slow for practical application, other degrees of dilution must be tested until the minimum dose is found which will kill the micro-organisms in a short time (*e g*, in thirty minutes)

Flasks of 15 c c capacity, provided with ground glass stoppers, are used for the test These are filled almost to the top with the disinfectant, and after a pure culture of each species has been placed in the flasks, they are thoroughly shaken

When the action is completed, every trace of the reagent must be removed from the vegetation by washing, and a sample of the growth is transferred to a suitable nutritive substratum, and exposed under the most favourable conditions It must be main-

tained at a constant temperature, which should be higher than that of the room Liquids are to be preferred to gelatine, because the nutritive value of the latter is generally smaller Finally, the observation of such growths must extend over a considerable period, as it often proves to be the case that the cells have not been killed, and after a considerable time they may germinate again The degree of dilution at which an antiseptic operates restrictively on species is usually dependent on whether the action takes place in a nutritive fluid or not In the first case, the chemical nature of the liquid obviously has considerable influence Thus, for instance, liquids which are rich in albuminoids weaken the effect of many poisons In research work of this kind, relating to practical fermentology, it is often necessary to use a definite liquid

As an example of the part that the solvent plays, the classical work of Koch in 1881 may be mentioned, which led to the proof that many antiseptics may wholly or partly lose their power according to whether they are dissolved in water, in ethyl or methyl alcohol, ether, or acetone In this connection an important fact may be noted The addition of sodium chloride to certain antiseptics (*e.g*, to carbolic acid or mercuric nitrate solution) causes an extraordinary increase in their antiseptic power

Temperature, also, has an influence on their action, the higher the temperature, the greater their activity. On the other hand, a dilute antiseptic exhibits the least restrictive power at that temperature which is most favourable to the organism

Numerous investigations regarding the influence of antiseptics on different species of micro-organisms have shown that no general rule can be traced One species may be much more resistant to one poison than many other species, whereas it may exhibit little resistance to another poison The destructiveness of a given substance cannot, therefore, be defined in general, but only its behaviour towards a particular species

Bokorny gives an explicit account of the results obtained by himself and others regarding the restrictive action of chemical substances on the growth of fungi He confirms the exactness of the observation that bacteria are generally far more sensitive to the action of acids and zymogenic fungi much less sensitive to alcohol, than are other fungi In order to show how very different are the actions of various poisons on yeast, Bokorny used 10 g. of commercial, pressed, brewers' low-fermentation yeast, which he dissolved in water in flat dishes, so that the yeast, after settling on the bottom, was covered by rather a thin layer of water He found the yeast was killed by the following doses —

Mercuric chloride,	0 01 to 0 005 g
Zinc sulphate,	0 1 g
Manganese sulphate,	0 3 to 0 5 g
Lactic acid,	0 05 to 0 1 g
Formaldehyde,	0 05 g

The common poisons are grouped by Loew as follows —**Oxidising** poisons, such as ozone, hydrogen peroxide, chlorine, phosphorus, etc, **catalytic**—*e g*, chloroform, ethyl ether, those acting by formation of **salts** acids, soluble mineral bases, salts of heavy metals, **substituting** poisons, destroying the plasma in consequence of their action on the aldehyde or amido groups of the active proteid substances—*e g*, phenols, prussic (hydrocyanic) acid, nitrous acid, aldehydes As special poisons may be mentioned toxic proteids, organic bases which combine with active albumen and thus are apt to destroy the structure—*e g*, strychnine, chinine, nicotine; indirectly acting poisons which hinder respiration, such as carbonic oxide, or cause decomposition (nitrites), or lastly alter the state of tension (oxalates, neutral salts of alkalies)

The application of antiseptics for the cleansing of vessels, etc, must always be preceded by a thorough mechanical cleaning

Amongst the mineral antiseptics, the first place must be given to **mercuric chloride,** on account of its extremely poisonous character. It is used in the laboratory in a solution of 1 gramme per litre of water, but it is impossible to use it in the fermentation industry Like most of the other mercuric salts, mercuric chloride belongs to that class of bodies which produce insoluble compounds with albuminoids, and thus do not react completely with bacteria Attempts have been made to overcome this difficulty in such cases by the addition of a small percentage of sodium chloride **Hydrofluoric acid** and its salts also belong to the most powerful antiseptics, especially as regards bacteria **Ammonium fluoride** is generally used, and has a wide application **Chlorine** is used in the fermentation industry in the form of chloride of lime, but it is only applicable within certain limits, owing to its strong and pungent odour Another compound, sodium hypochlorite (**antiformin**), which has a weaker smell of chlorine, is more widely employed Chlorine is also used to disinfect water For this purpose small quantities of chloride of lime are used, and after a short time the chlorine is fixed by the addition of calcium bisulphite **Sulphurous acid** is applied sometimes in the form of gas or of an aqueous solution, and sometimes as calcium bisulphite or sodium sulphite It is used, not only as an antiseptic, but also as a means of restricting fermentative activity This, as well as several of the above, usually appears to attack bacteria more strongly than yeasts in high dilutions * **Soda** is of value as a means of cleansing, as well as disin-

* Regarding the use of **sulphurous acid** and **potassium metasulphite** in grape and fruit wine, a large amount of research work was done by Muller Thurgau, both in the way of regulating the fermentation and preventing it in grape and fruit juices On addition of the acid in the course of fermentation—preferably in the early stages—part of it combines with the aldehyde present and is rendered inactive, a further portion forms glucose-sulphurous acid, which gradually splits up during fermentation, the glucose being fermented and the acid set free In the later stages, a greater or less proportion will be found free, and the remainder either combined or oxidised to sulphuric acid The quantity to be added depends partly on the composition of the juices, which is very

fecting, and this applies also to **lime** Lastly, two strong oxidising agents must be mentioned which are now in common use, **ozone** and **hydrogen peroxide,** the latter having an even greater disinfecting power than ozone

Amongst organic antiseptics, **formaldehyde** has found very extended application during the last few years, on account of its great antiseptic power Thus, the spores of many resistant bacteria are killed by the application of a 0 1 per cent solution for an hour On the other hand, this reagent, when used in the form of vapour, has little action on man and the higher animals Its vapours appear principally to attack the surface of articles, as its power of penetration is not great

A series of antiseptics which have proved of special importance in laboratory studies of fermentation includes **ether, chloroform,** and more especially **acetone, toluol,** and **thymol,** because they possess the valuable property of killing germs, while they do not destroy enzymes This fact has proved of importance in advancing recent studies of enzymes, where it is necessary to inhibit the action of micro-organisms on the susceptible liquids employed. R Koch first proved the antiseptic action of ethyl alcohol, and recent research has brought to light the interesting fact that it is not absolute alcohol, but a 50 to 60 per cent alcohol that exhibits the strongest disinfecting power This may be explained by supposing that absolute alcohol absorbs moisture from the surface of the cells, and, therefore, makes them more resistant The vapour of 75 per cent alcohol appears to be equal in its action to a current of steam, and a still more powerful action is exhibited by a mixture of alcohol vapour of this strength with formaldehyde vapour The mixture may be used to disinfect fabrics which would suffer by exposure to a temperature of 100° C

Carbolic acid (phenol), which plays an important part in medicine as a powerful antiseptic, cannot be applied in the fermentation industry, owing to its penetrating odour, but owes its interest to the fact that it does not attack enzymes On the other hand, a whole series of compounds, of which carbolic acid is a component, are made use of in practice

variable, especially that of fruit juices, and partly on the differing power of resistance of yeasts In Muller Thurgau's experiments, the doses were found to range from 225 to 600 mg of potassium metasulphite per litre of grape wine, corresponding to about 100 to 300 mg of sulphurous acid When selected races of pure yeast are used, it will be necessary to acclimatise them to the acid, especially when the object in view is to check the bacteria (Chap vi) To prevent the biological decomposition of malic acid, only slight doses of acid are required, as the bacteria concerned are very sensitive to its influence, the acid has then to be employed *before* fermentation If the object is to prevent the formation of lactic acid in fruit wine of low acidity, it is necessary to use larger proportions in the initial stages of fermentation The best means of preserving grape and fruit juices can only be determined by preliminary experiments in each case If the juice contains large quantities of tartaric, malic, and tannic acid, smaller doses of sulphurous acid will do Thus, in one instance, fermentation was completely prevented by an addition of 225 mg of potassium metasulphite (120 mg sulphurous acid) per litre

The raw materials of the fermentation industry (rye, wheat, barley, etc) contain peculiar compounds which, according to the researches of Jago, Delbruck, Lange, Henneberg, Hayduck, and Seyffert, act as poisons to yeast, and these are assumed to be of an albuminoid character This action may be observed in the crushed grain or in an aqueous extract if the yeast is added in presence of sugar dissolved in distilled water.

In their reaction to such influences, the yeast species do not behave uniformly Thus, under certain conditions a stimulus may be given to some species, whilst under other conditions the poisonous substance may act destructively even in minute doses. Such is the case with the poisonous body present in rye bran, and also with that contained in a dilute hydrochloric acid (0·1 per cent.) extract of wheat flour, in their action upon brewer's yeast; but the action is arrested by the addition of calcium carbonate, soda, gypsum, etc

As stated above, it has been proved that minute quantities of poisons may have such an influence on micro-organisms that they actually stimulate them, often to a one-sided growth, it may be the development of the vegetative organs at the expense of the organs of propagation, or an increase of the fermentative activity. In a few cases that have been closely examined it has been proved that the minute doses which can produce such an action have fairly well-defined limits, the least excess brings about the opposite action—a weakening of the organisms in question. Thus a minute dose of a copper salt assists the development of the mould, *Aspergillus niger*, to a very great extent In the same way Biernacki found that the addition of copper sulphate in the proportion of 1 . 600,000 of the nutritive liquid increased the activity of yeast cells In larger quantities copper salts exercise a destructive action on yeast, care must, therefore, be taken that when pure cultures of yeast are introduced into copper vats, these should be carefully tinned Hayduck (1881) found that small quantities of lactic acid (about 0 5 per cent) favour the propagation of yeast, and that anything up to 1 per cent. of lactic acid, under the usual technical conditions, is favourable to the production of alcohol Heinzelmann proved in 1882 that salicylic acid in the proportion of 0 1 gram per litre reacts favourably on yeast cells, and H Schulze (1888), that minute traces of poisons, such as mercuric chloride, iodine, chromic acid, and formic acid, have the same action (*e g.*, mercuric chloride in a dilution of 1 . 500,000). Hirschfeld found that by the addition of 0·01 to 0 02 per cent. of hydrochloric acid the acetic fermentation is very considerably quickened. Richet proved that the same holds good with lactic acid bacteria, while the addition of 0 5 mg. of mercuric chloride, or of copper sulphate, per litre, intensifies their fermentative activity In the same way Effront found that minute quantities of

hydrofluoric acid and fluorides have a stimulating action in nutritive liquids, both on the rate of propagation and the fermentative capacity of yeasts, but that this varies with the yeast species.

The fungi have a curious power of accommodating themselves to poisons. By long-continued cultivation it has proved possible to introduce large quantities into nutritive substrata, and at the same time it has been noted in several cases that a marked change of character takes place It has, however, proved impossible to fix these newly acquired characters, they are of a purely transitory kind As soon as the poison is removed the growth reverts to its original character. From the numerous examples, we select the following:—Galeotti accustomed *Bacterium prodigiosum* to grow on a 2 per cent carbolic acid nutritive gelatine Pulst accustomed *Penicillium glaucum* to withstand continually increasing quantities of poisonous copper salts, whilst its conidia germinated more rapidly than usual.

The results obtained by accustoming yeasts to the presence of certain poisons are of special interest in the technology of fermentation Thus, the yeast in distilleries may work in a mash which by treatment with a disinfectant has been rendered more resistant to bacteria, a process which takes the place of the usual souring with lactic acid For this object sulphuric acid, hydrochloric acid, and hydrofluoric acid have been made use of Effront proved that much smaller quantities of hydrofluoric acid were required than of the other two In consideration of the different extent to which the yeasts are attacked by the hydrofluoric acid (or fluorides), Effront tried by special cultivation of yeasts to accustom them to work in a mash which contained so much of the reagent that the bacteria were suppressed. He found that the addition of 300 mg of hydrofluoric acid to 100 c c of liquid completely inhibited the growth of yeast, whilst its fermentative activity was only restricted If, however, the yeast is gradually accustomed to the poison, beginning, for example, with 20 mg per 100 c c., and rising by degrees to greater doses, a race of yeast will be formed that can multiply even in presence of the original dose In presence of 200 mg per 100 c c the fermentative power of the yeast is increased, according to Effront, if it is introduced into a mash which also contains fluorides Yeast acclimatised to a fluoride will convert that absorbed by the cell into insoluble calcium fluoride, and thus render it innocuous The higher the yeast is acclimatised, the larger will be the amount of calcium in its ash In practice about 10 grams of hydrofluoric acid are used for every hectolitre of mash Even if this process succeeds in suppressing bacteria in the mash, which is not always the case, other difficulties may arise when wild yeasts are present, for these, according to Holm and Jorgensen, are stimulated in their development by the presence of hydrofluoric acid in the mash

4. Disinfection in Practice.

It has become clear, within the last few years, that the harmfulness of the germs in air and water has been greatly exaggerated, and that far and away the most important source of danger is to be sought in the growth of foreign organisms in the plant itself The natural result is that increasing attention is being paid to a rational scheme for disinfecting the plant As the raw stuffs used in breweries, distilleries, etc, form an admirable nutritive medium for micro-organisms, the distribution of these throughout the plant is much more widespread than usual, and it is often necessary, in addition to mechanical cleansing, to attack them by direct antiseptic means By determination, on the one hand, of the maximum limit of such poisonous substances that can be allowed, and, on the other hand, of the necessary means to secure the desired object, the practical conditions are established. The concentration must not exceed what is absolutely necessary In the use of antiseptics it is essential to follow a recognised plan A summary disinfection is insufficient if the individual parts present different possibilities for the development of foreign organisms It is, therefore, necessary from time to time, and that frequently, to overhaul every single point in practice, before being able to say exactly where a particular infection has appeared At certain points antiseptics must be discarded and mechanical means adopted This is the case when the infection has penetrated so far into the material that the disinfectant is no longer able to attack it This may occur in the great majority of wooden vessels as they are usually prepared

As many micro-organisms form slime, and may produce thick deposits when allowed an undisturbed development, it is often necessary to use a solvent of the slime before proceeding to actual disinfection, if the germicidal substance is not capable of completely dissolving the slime

It is an established rule that two disinfectants should not be used simultaneously, or one immediately after the other, especially if their composition is unknown, otherwise there is danger that they may neutralise each other's action Thus, chloride of lime and calcium bisulphite should never be used at the same place

The literature of antiseptics used in the industry has grown to considerable proportions A short *résumé* of the methods of application of the respective substances follows

Ammonium fluoride, especially the acid salt, has a very wide application, owing to its great antiseptic power It is a crystalline powder, readily soluble in water. In the pure condition it contains about 35 per cent. of hydrofluoric acid, the commercial product, however, contains a less quantity, and sometimes not more than 20 per cent. It is used for the treatment of pipes, vats, etc Pipes are filled with a solution containing about 0·5 per cent In rinsing

out vats a 3 to 5 per cent. solution must be used. Ammonium fluoride is not suitable for the treatment of metal, as it slowly attacks it After treatment, a very thorough washing with water is necessary

Formalin has also been very largely applied in practice. It is an aqueous solution of formaldehyde (40 per cent. by volume or 37·2 per cent by weight), and it forms one of the most powerful antiseptics As it does not attack metal, it can be applied more generally than the fluorides. It may be used in the form of gas by soaking cotton wool or cloths in formalin, and hanging them up in the area to be disinfected, or it may be applied in specially constructed lamps, in which, by the imperfect combustion of methyl alcohol, formaldehyde is produced The most frequent and most successful method of application is, however, to dilute formalin with water, and apply it as a spray to the walls of vats, etc. A solution of 0 5 per cent of formaldehyde (about 1½ litres of the commercial article to 100 litres of water) is most generally applicable The vessel must then be well rinsed with water, and if the odour cannot be got rid of, ammonia may be applied

Chloride of lime has been used for many years, on account of its powerful disinfectant properties Its strong odour limits its application It is especially used to disinfect floors and slimy walls in rooms where fermentation is going on. To disinfect filter bags in breweries, which often harbour large colonies of bacteria and wild yeast, Will recommends an application of chloride of lime in a solution containing about 1 per cent of active chlorine (3 to 3½ kilos chloride of lime in 1 hectolitre of water) The mixture of water and chloride of lime is allowed to stand for some time, with occasional stirring, the clear liquid is then decanted and applied to the filter bags, which are afterwards repeatedly rinsed with water The dangerous development of micro-organisms on the filter bags may be avoided by cooling down the beer to the lowest possible temperature during filtration

Antiformin is a chlorine preparation which has found considerable application in recent times It is a clear liquid with a strong alkaline reaction and a weak odour of chlorine. It consists of a crude sodium hypochlorite (*cf* Eau de Javelle), and is prepared by decomposition of chloride of lime with soda The solution is then separated from the precipitated chalk, and caustic soda is added The liquor contains more than 4 per cent of active chlorine, and not only possesses great antiseptic power, but also quickly softens organic substances such as sediment, wort, crust, yeast, and slime, so that they can easily be removed. In other words, it acts both as a cleansing and as a disinfecting agent Care must be taken, however, in applying it to infected wood, for instance, to the staves of a fermenting vat, as the reagent, owing to its solvent power, can penetrate so far that it is difficult to remove

it by rinsing with water It may be applied in a dilution of 1 to 20. *Bichlorin*, *Radoform*, and *Mikroformin* are more recent antiseptics of similar composition.

Antigermin appears to be specially adapted for washing down walls. It consists mainly of a copper salt of an organic acid, and the aqueous solution is without smell It should be dissolved in boiling water, and mixed with lime before applying.

Montanin, which is also free from smell, is equally applicable to walls and to connecting pipes, vats, etc, but the latter must always be well rinsed It is a by-product of the glazed-ware industry, and contains about 28 to 30 per cent of hydrofluosilicic acid (as aluminium fluosilicate) in a clear solution, pale green or yellow in colour, and feebly acid The protection of walls by means of this preparation depends upon the pores being sealed by the formation of calcium fluoride, alumina, and silica, imparting to the wall a hard and smooth surface

Mikrosol appears in commerce as an acid green paste containing about 10 per cent of copper phenolsulphonate, and smaller amounts of copper sulphate, free sulphuric acid, and hydrofluoric acid It is applied to walls in the form of a 2 to 4 per cent solution

Antinonnin is largely used in order to coat moist walls, and is an excellent preventive of dry-rot, and protects woodwork from worms, etc It forms a red viscous mass consisting of a potash compound of cresol mixed with glycerine, soap, etc It is soluble to the extent of 5 per cent in water It does not attack either metals or organic substances, and, according to Aubry's investigations, may be applied to advantage throughout the brewery, where it cannot come in contact with beer

Pyricit is a white powder soluble in water to form a colourless and odourless solution According to Wichmann, a 2 per cent. solution forms a very powerful disinfectant. It may be applied anywhere, inside or out It can be kept for a long time without losing its activity The powder is a mixture of sodium bisulphate, sodium borofluoride, and sodium fluoride On dissolving in water several acids are set free, among which hydrofluoric acid is the most important and active Metals are but slightly attacked, if at all.

Sulphurous acid is one of the oldest antiseptics, and is still frequently used for casks A piece of linen which has been dipped in molten sulphur is set alight and introduced into the cask. The fumes do not, however, penetrate sufficiently to sterilise badly contaminated casks Hops and occasionally malt are also treated with burning sulphur. In wine fermentation sulphurous acid is added to the must, to destroy the spontaneous germs before adding pure wine yeasts **Calcium bisulphite** forms an energetic reagent; the solution usually contains about 7 per cent of sulphurous acid. Diluted from three to six times with water, it forms an admirable

agent for the treatment of vats and other apparatus, and is especially deadly to moulds.

Salicylic acid has also been applied to vats in the form of a dilute alcoholic solution, which is painted on to the surface, allowed to react for some time, and then washed off with an alkaline liquor, and finally with water

Amongst weaker antiseptics, lime and soda may be mentioned **Milk of lime,** freshly prepared, forms an excellent disinfectant for walls and ceilings, but as soon as the lime has absorbed carbon dioxide from the air it ceases to have any value **Soda,** in the form of a 5 to 10 per cent solution in warm water, is an excellent reagent for dissolving slime from connecting pipes, etc It must, however, be very thoroughly removed by washing, first with warm and then with cold water. A very dilute soda solution (0·1 to 0 3 kilogramme per hectolitre of water) is of value in swilling new chips. Soda is not well adapted for disinfecting fermenting vats, as it imparts a rough surface to the wood.

One of the most important disinfectants throughout the fermenting plant is **steam,** if care be taken that every part of the vessel to be treated is exposed to its action Connecting pipes may be sterilised by steam if they do not exceed a certain length

In distilleries, **sulphuric acid** is used as a disinfectant in the mash to inhibit the growth of foreign bacteria, and to restrict that of yeast Its application must, however, be kept within certain limits, as the yeast would otherwise be damaged

Ozone has found application, in particular for disinfecting water In order to bring the gas into close contact with water, the latter is sprayed over a fine-grained material, where it comes in contact with a stream of ozone prepared by means of a high tension electric current, discharged from two electrodes of special construction It has proved possible by this means to kill a very large proportion of the organisms in water (see the researches of Calmette, Schuder, and Proskauer, Ohlmuller, etc.). The solubility of ozone in water depends largely on a sufficiently high concentration of ozone in the medium from which the ozone is carried into the water Its solubility is enhanced by a low temperature and slight acidity of the solvent Water containing 15 to 20 mg of ozone per litre is a powerful disinfectant of filter cakes, transport vessels, etc, if left to act for a sufficient time According to Will and Weissinger, the noxious germs in a brewery are killed in an hour by 0 6 to 0·7 gram of ozone per cubic metre of *air*.

Hydrogen peroxide has also been applied to disinfecting water, and preserving milk by Budde's process, which consists in the application of 0·036 per cent, after which the milk is maintained for three hours at 50° to 52° C

5. Flasks.

All vessels in which cultures are made must satisfy the condition that they are proof to every contamination from without **Pasteur flasks** satisfy this demand in the highest degree The illustration (Fig 5) shows this flask in the improved form employed in the author's laboratory When the hopped wort (preferably filter-bag wort) is boiled, the steam first escapes through the straight tube, attached to which is a short piece of rubber tubing, when this is closed (after boiling for about half an hour) the only outlet for steam is through the bent tube About twenty minutes later, the flask is removed from the sand-bath During cooling the germs are deposited in the lowest part of the bent tube, or are not carried beyond the enlargement of the tube, and, therefore, do not come into contact with the liquid Hence, it is evident that the lower part of the bent tube must be heated whenever the flask is to be agitated or emptied through the straight tube If the flask is to

Fig 5 —Pasteur's flask

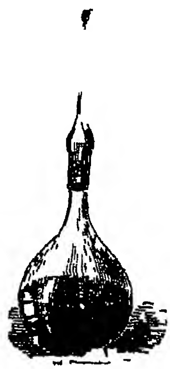


Fig 6 —Chamberland flask.

be opened and connected with another flask, this must be effected either in a small sterile space, or else the opening and connecting must be carried out in a flame A Bunsen burner is placed directly in front of the operator, the flask to be emptied to the *left*, and the one that is to receive the liquid or culture to the *right*, alongside the burner. Then the tube of the left-hand flask is opened *in the flame* by quickly removing the rubber tube with its glass stopper, while the open tube is in the flame, the glass stopper of the flask to the right is quickly withdrawn, and the hot tube of the first flask is connected with the rubber tube of the second flask after the tube has been cooled. The liquid is poured into the second flask, the bent tube of the first flask being at the same time heated. Then the side tube of the left flask is again introduced into the flame, while the stopper of the right flask is replaced directly after is has been passed through the flame, finally, the left flask is

closed *in the flame* with its tube and stopper When the operation is quickly performed the danger of contamination is almost excluded.

Pasteur flasks will be found indispensable in certain operations; for instance, in physiological researches where large quantities of liquids are dealt with

The **Chamberland flask** (Fig 6) is closed with a ground cap, which terminates in a short, open tube, this tube is filled with tightly-packed sterilised cotton-wool

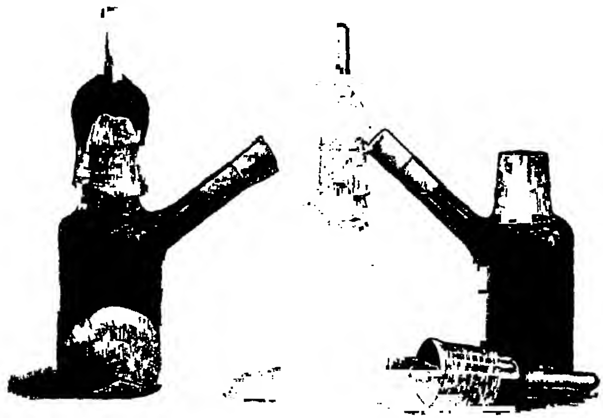


Fig 7

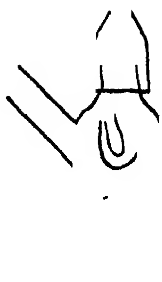


Fig 8

Fig 9

The **Freudenreich flask** is constructed on exactly the same principle (Fig 7, centre), it has, however, a cylindrical shape

These flasks must only be opened in a sterile cupboard When gelatine is used the flask must be opened with the mouth downward.

For special purposes the **Hansen flask** (Fig 7, left) is employed The ground cap is provided with a cotton-wool filter, and the flask has a small side-tube closed with an asbestos stopper This flask is used in the author's laboratory for the dispatch of small cultures

or of samples from the propagating apparatus.* For this purpose the lower part of the flask is filled with cotton-wool, and some cotton-wool is lightly packed into the cap. The asbestos stopper and the lower edge of the cap must be covered with sealing wax.

A flask (Fig. 7, right, and Fig. 8) constructed by the author carries a small, bent, and open tube in the cap, as a prolongation

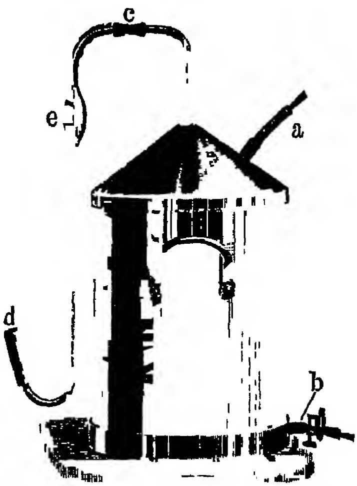


Fig. 10.—Carlsberg flask—Old model.

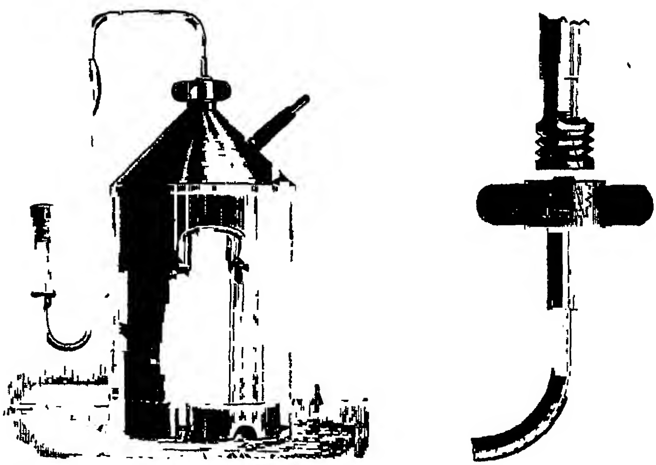


Fig. 11.—Carlsberg flask—New model. *b*, Connection between the flask and the bent tube.

of the cotton-wool filter. By this means it has proved possible to prevent the evaporation of the contents of the flask for several years, provided that the lower edge of the cap and also the lateral tube are well closed. This flask is used for preserving pure cultures

* This apparatus is described in Chapter VI.

of yeast in a 10 per cent. saccharose solution inoculated with a trace of the yeast The flask is also suitable for a prolonged preservation of gelatines, if the surface is to be prevented from stiffening Another flask constructed by the author, shown in section in Fig 9, is also used for storing pure cultures of bottom and especially of top yeasts which will not stand vigorous shaking, when further development is required and a drop of the yeast is to be transferred to a Pasteur flask containing wort. The small bent open tube on the right has its outer extremity packed with cotton-wool, to filter incoming air. The wide tube on the left, which is closed with an asbestos stopper, has its lowest bend on a level with the bottom of the flask. If this tube is connected with the side tube of a Pasteur flask in the flame, and then suction applied to the bent tube of this flask, a minute part of the yeast lying on the bottom of the small flask will be sucked into the Pasteur flask, without disturbing the remainder of the yeast deposit If it is necessary frequently to remove a small portion of a culture, this process may be recommended

For the development of very large cultures the Carlsberg vessels (Figs 10 and 11) are employed They have a capacity of 10 litres, are made of tinned copper, cylindrical in shape, and conical at the top ; at the apex of the cone a twice-bent tube (*c d*) with or without an enlargement (*e*) is soldered into the flask At one side of the cone is the inoculating tube and glass stopper (*a*), and near the bottom of the vessel is another tube (*b*) for drawing off the fermented liquid and the yeast This tube is provided with a pinch-cock When the liquid is sterilised, the bent tube is closed with an asbestos or cotton-wool filter, which is tightly packed on to the end (*d*).

In the new model (Fig 11) the bent tube is ground into the upper part of the flask, and fastened by means of a screw, allowing the whole of this part to be detached, when the flask is to be cleaned ; the filter is screwed into the end of the bent tube.

6 Nutritive Substrata.

With regard to the nutritive substrata, the problem naturally consists in finding those which are best suited to the respective organisms If they also possess the advantage of being less favourable for the development of competing forms, it is a great point gained The fact must, of course, be borne in mind, when comparative investigations are made in different directions, that the nutritive liquid must always remain the same

For the investigation of yeasts, hopped beer-wort forms t[he] best nutrient It is best taken from the filter-bags, because t[hey] yield a smaller deposit on boiling in the flasks It is also suit[able] for many bacteria and moulds; but for certain bacteria (*e g.*, l

acid bacteria) the sweet wort, without hops, is used, and this is also adapted for use with moulds. Amongst the artificial nutritive fluids for yeast, Pasteur's (1860) has an historical interest. The renowned scientist used it to upset Liebig's theory with regard to the indispensability of albuminoids for fermentation. It consists of 100 c c of water, 0 075 g of the ash of yeast, 10 g of sugar, and 1 g of ammonium tartrate. A good nutritive fluid can also be prepared from yeast decoction with 5 to 10 per cent of sugar. Yeast decoction is an aqueous extract of yeast (about 1 litre of yeast to 2 litres of water, boiled under pressure), filtered, and either neutralised or rendered slightly alkaline with sodium carbonate or lime. For special research, compound liquids may be used containing sugar and the salts necessary for nutriment and normal growth of yeast, including potassium, magnesium, and calcium, phosphates and sulphates For further details, see the chapter on the Nutrition of Yeast

Special nutritive liquids are also used for bacteriological investigation Cohn's solution has historical interest, and its composition is as follows —100 c c of water, 0 5 g mono-potassium phosphate, 0 05 g tri-potassium phosphate, 0 5 g crystallised magnesium sulphate, and 1 g ammonium tartrate To-day a *nutritive broth* is chiefly used, prepared by steeping finely chopped beef for a few hours in water, and then boiling and filtering the liquor The liquid is generally neutralised with soda, or rendered slightly alkaline, and after adding 1 per cent of peptone and 0 5 per cent of sodium chloride, it is again boiled, filtered hot, and finally sterilised in flasks or test tubes. Such an extract must obviously vary in composition, and in special cases resort may be had to an artificial nutritive liquid, free from albumen We may quote that prepared by Voges and Proskauer, consisting of 1 litre of water, 5 g sodium chloride, 2 g disodium phosphate, 6 g. ammonium lactate, and 4 g asparagin

A Fischer's base consists of 0·1 per cent of di-potassium phosphate, 0 02 per cent magnesium sulphate, and 0 01 per cent. calcium chloride, dissolved in tap water The solution is then added to peptone, or peptone and sugar, etc , according to the requirements of the particular species of bacteria For the development of lactic acid bacteria (from milk) O. Jensen uses peptonised milk prepared by treating 1 litre of milk with 10 c c of hydrochloric acid and 2 g of pepsin By keeping it in the thermostat and frequently shaking, the precipitated casein is redissolved, the liquid is then neutralised, cleared with albumen, and sterilised at about 120° C

For moulds, in addition to beer-wort, fruit decoctions and sugar solutions containing tartaric acid and tartrates are used, or, again, the complicated Raulin's liquid, which is also applicable to bacteria, and consists of—water 1,500 c c, sugar 70 g, tartaric acid 4 g.,

ammonium nitrate 4 g, ammonium phosphate 0 6 g, potassium carbonate 0 6 g, magnesium carbonate 0·4 g, ammonium sulphate 0 25 g, zinc sulphate 0 07 g, ferrous sulphate 0·07 g, potassium silicate 0 07 g

If solid nutrients are required, 5 to 10 per cent. of gelatine is added, or, in the case of cultures which are to be developed at or above 30° C, about 1½ per cent of agar-agar, a jelly derived from salt water algæ, and, by contrast with gelatine, containing only a minimum of nitrogen For the cultivation of thermophilous bacteria at 60° to 70° C, Miquel uses carragheen moss instead of agar, in the proportion of 2 to 3½ per cent Slices of potato sterilised in an autoclave are often used as a solid nutrient Black bread makes an excellent solid substratum for moulds For the cultivation of the nitrifying bacteria Winogradsky and Omeliansk used gelatinous silicic acid.

For plate-cultures of acid-forming bacteria (lactic acid and acetic acid bacteria) some litmus or, preferably, according to Beijerinck, carbonate of lime (finely precipitated chalk) is added The gelatine thus acquires a motley appearance, but the colonies of acid bacteria are surrounded by a clear zone, because the acid dissolves the chalk By the use of zinc carbonate in plate-cultures, the acetic acid bacteria form colonies and display clear zones, whereas the lactic acid bacteria are relatively sensitive to this salt, and their growth is inhibited

Pasteur used liquids exclusively for his work on the ferments. Later, solid media became of great importance, and Koch laid the foundation for their application.

Plate-cultures are prepared by introducing the growth into the liquefied gelatine, and then pouring the mixture into a Petri dish. When the gelatine solidifies the individuals are separated throughout the mass, and, on development, they appear as colonies, visible to the naked eye *Streak-cultures* are those in which a minute portion of the growth is introduced on to the surface or into the upper layer of the solidified gelatine on a platinum spatula *Stab-cultures* are those in which a fraction of the growth is introduced by an inoculating needle into a thick layer of solidified gelatine *Giant colonies* are formed by pouring a drop of the inoculated liquid on to a stab in the solid gelatine

Comparative researches on colonies of this kind formed by various types of budding fungi were made by Lindner and Will. By means of a fine pipette a tiny drop of yeast is sown upon a layer of wort or potato-water gelatine, 2 cm thick The same cell forms gradually appear as in the films formed on the surface of liquids, but the growths acquire a different macroscopical appearance The exhaustive investigations of Will, in particular, showed typical differences to exist between the two great groups of budding fungi, when grown for a sufficient length of time The

true saccharomycetes develop, not only on the surface of the gelatine—where clusters of elongated cells radiate from the edge of the central region—but the cells are also seen to grow down into the gelatine, either uniformly or in closely packed but not equally distributed clusters of cells, or with wart-like or clustered appendages, completely enclosed, radiating from the central region, growing upwards, and sometimes outgrowing the border of the colony In these stages of development the colony appears more or less variegated with radial stripes and concentrically elevated zones, while the middle region also assumes different shapes, either flat or raised like a crater above the rest of the colony. A different mode of development is observed in the second group of budding fungi—Willia, Pichia, Mycoderma, Torula—where the growth generally takes place only on the surface of the gelatine. The radial and concentric corrugations, which are often rather indistinct, are here due to displacement caused by lateral pressure produced by the more freely growing cells Between these "fundamental forms" of the two groups there exist many transitional forms

For the **preservation** of pure cultures agar, gelatines, or liquid media are used. Owing to the great liability to variation which is characteristic of **bacteria,** it is generally necessary frequently to renovate their cultures to avoid any alteration taking place in their physiological condition The lactic bacteria used for industrial purposes are preserved in milk or mash, and must be renovated at short intervals * Most **mould** fungi will stand prolonged preservation on agar or gelatine. In dry form (in layers of filter-paper) many mould species can be kept unimpaired from year to year. For the preservation of **yeasts**, wort-gelatine, wort, or a 10 per cent aqueous solution of saccharose is employed The experience of Hansen and the author, and later that of Holm and Klocker, proved that the saccharose solution is an excellent medium for preserving yeasts, in which they can be kept alive for many years In the author's laboratory the most satisfactory results in preserving specific properties intact have always been obtained when the saccharose solution was inoculated with a very small dose of yeast Wort-gelatine, or more particularly wort, may be employed when yeasts are to be preserved for a long time, nevertheless, in the author's experience, the use of wort media involves the risk, as far as culture yeasts are concerned, that certain races may lose the properties most highly appreciated in practice (at all events for a considerable time)—*e g*, in point of attenuation and power of

* For the preservation of the lactic acid bacteria of milk, Jensen uses milk saturated with chalk or, if they are to be preserved for a greater length of time, agar mixed with a slight proportion of dextrose and casein-peptone prepared in the following manner — 100 g (sugar-free) acid-casein is digested for a week at blood temperature with 1 litre water, containing 4 6 per cent HCl and 2 g. pepsin The solution formed contains, after neutralisation, sterilisation and filtration, about 1 per cent N and 1 2 per cent NaCl

clarification. Some species, such as Sacch. Ludwigii and the Schizosaccharomycetes, cannot live in saccharose, but remain alive in wort for many years

7. Preparation of the Pure Culture.

To prepare an absolutely pure culture, it is necessary to make sure by direct observation that the development begins with a single cell, and that this is so completely isolated that during the development no other cell can creep in and render the culture impure If such a pure culture is required for experiments on a large scale or for actual fermentations, special rules must be observed in order that the absolutely pure growth at first developed shall be protected from every infection during its further growth in a succession of larger flasks Care must, of course, be taken that the species is developed under the most favourable conditions to secure a vigorous and normal culture The process in its later stages is described in another section We are here concerned with the problem of securing the first absolutely pure culture as the point of departure for the mass culture

The desideratum of direct observation presents difficulties in the case of the smallest micro-organisms—bacteria. Whilst it has long proved possible to directly observe single cells of yeasts and moulds on account of the size of their cells, this was not the case with the great majority of bacteria It is only recently that the technique has been sufficiently developed to allow of an accurate solution of this problem

Long before there was any attempt to work experimentally with absolutely pure mass cultures, experiments in the cultivation of micro-organisms had been undertaken with a purely botanical object, to discover what different forms a species may assume, and with this object the development of single cells was followed under the microscope

As early as 1821, Ehrenberg observed the germination of the spores of certain fungi by careful observations of this kind The propagation of yeast cells was observed by Mitscherlich (1843), Kutzing (1851), and F Schulze (1860), in the same way A small quantity of top yeast was diluted with beer-wort until a drop contained only one or two yeast cells , from this drop an ordinary preparation was made, the cover-glass was cemented on to the glass slide, to prevent evaporation, and the development of the cell was watched under the microscope Similar cultures were employed by Tulasne (1861) and de Bary (1866), in their famous researches on the germination of spores A considerable improvement in the method was made by Brefeld during his detailed researches on mould, blight and mildew fungi, in which he followed the development of the mycelium until it, in its turn, again formed spores The infection

on the object glass was protected by means of a small shield of paper fastened on to the tube of the microscope, and this was afterwards converted into a moist chamber (1881), after Brefeld had recognised the danger of foreign germs penetrating into the cultures He diluted the material with water, brought a drop containing a single germ on to the cover-glass, added some nutritive liquid containing gelatine, and placed the cover-glass, with the drop underneath, on to a glass ring (Bottcher's chamber), which was fastened to the object glass As the apparatus and the nutritive liquid were sterilised, all the necessary conditions were fulfilled for carrying out a culture experiment without contamination We may here see how improved methods of cultivation have led to the preparation of an absolutely pure culture By the help of his cultures Brefeld (1883) made the interesting observation that in quite a number of fungi (*e g*, the smut of wheat, the boil-blight of maize) the conidia are able to propagate by direct budding, like yeast, without throwing out new seed-carriers.

A short survey follows of the different methods which have been applied for preparing *pure cultures on the large scale.*

(*a*) **Physiological Methods.**—At the earliest stage, attempts were made to reach the goal by calculating probabilities, and treating the whole growth, without condescending to isolate single cells

The physiological methods, "the enriching process" employed by Pasteur, Cohn and others, start with the fundamental idea that the various species occurring in a mixture will multiply unequally according to their different nature when they are cultivated in one and the same nutritive liquid and at the same temperature, so that those species for which the conditions are unfavourable will be gradually suppressed by the one or more species for which the conditions are favourable When the growth has developed under the selected conditions for quite a short time, a minute fraction is inoculated into the same nutritive liquid in a fresh vessel at the same temperature, and this process is repeated many times Different liquids have been employed for such cultures from time to time, for instance, alkaline liquids for bacterial growths, acid liquids to free yeast growths from bacteria (lactic, tartaric, hydrofluoric acids, etc.) The weak point of all such methods is, that they *start from an unknown material*—namely, *the impure mixture* It is, therefore, impossible to know what results such a treatment will lead to, for we are not dealing with any true method In fact, there is always the possibility that the weaker species are not destroyed, but merely checked and retarded, so that when the stronger species, after reaching their maximum development, become weaker, other species will have a chance of multiplying. This possibility also occurs when the growth is transferred to another substratum Likewise, there is always the possibility that not one but two or more species thrive equally well in the

liquid, and, consequently, develop to the same extent Such, for instance, was the case with brewers' yeast before pure cultures were employed. This yeast often yielded several typically different species of "culture yeast," as they are termed The method given by Pasteur for the purification of brewers' yeast may be quoted as a marked illustration of the dangers connected with the physiological method of treatment The impure yeast-mass is introduced into a cane-sugar solution, to which a small amount of tartaric acid has been added The object of the method is to free the yeast from any disease germs with which it may be infected. Hansen's investigations have, however, proved that, even if the bacteria are suppressed or checked by this treatment, simultaneously the wild yeasts, and among them those productive of diseases in beer, will develop abundantly, and in many cases the culture yeast, which it was intended to purify, is entirely suppressed. Even if there is primarily only a trace of the wild yeasts or "disease" yeasts, these are apt to develop to such an extent by Pasteur's treatment that they may eventually form the predominant part of the yeast-mass

The use of hydrofluoric acid or its compounds, such as ammonium fluoride, for the purpose of purifying an impure yeast—brewers' or distillers' yeast—as proposed by Effront, is liable to lead to the same dangers as the use of tartaric acid Methodical experiments made by Holm and the author have shown that by treating impure yeast according to Effront's process, the growth of wild yeast and Mycoderma species is forced more than that of the culture yeast, they have also shown that such a dangerous species as *Bacterium aceti* is in many cases not suppressed at all by the treatment in question, but, on the contrary, multiplies more rapidly in presence of hydrofluoric acid or fluorides

Phosphoric acid is also employed in practice for repressing bacterial growth, and it is claimed that the acid has the additional advantage of stimulating the alcoholic enzyme Some 2 to 3 g of acid per kilo are added to the yeast on pitching or from half an hour to an hour before pitching

If, now, we ask whether it is advisable to employ any of the various methods mentioned above for the purification of an unknown and impure yeast-mass, the answer must be in the negative, and this will be the case whether the culture is intended for purely scientific or for industrial purposes, for the danger will never be excluded that in prolonged cultivation other species than the one desired will gain the supremacy The starting point being uncertain, it necessarily follows that the result must be so too. In fact, all such methods must now be regarded as antiquated, and as complete failures Nevertheless, they may possibly be used in isolated cases before proceeding to the preparation of a pure culture In this way it is possible by suitable treatment

of the impure material to secure a preponderance of the group of the desired species in the mixture, so that pure cultivation may be facilitated Thus the treatment described with tartaric acid or hydrofluoric acid gradually converts the mixture into a growth of wild yeasts If a mass of yeast is strongly contaminated with bacteria, cultivation at very low temperatures may possibly suppress the bacteria. if the yeast is able to develop under these conditions If it is desired to obtain a pure culture of the lactic acid bacteria from a mash, the material may be prepared by keeping it a short time at 50° to 55° C. At this high temperature many bacteria cannot thrive, whilst certain species of lactic acid bacteria can stand a high degree of heat, and thus spread throughout the material In the same way in the cultivation of film-forming bacteria, such as acetic acid bacteria, the growth may undergo a preliminary purification by repeated inoculation of the film in fresh liquids. This process was used by Pasteur in his researches on acetic acid bacteria To make an approximate separation of a large and small species of yeast in a mixture, we may resort to decantation or filtration through a medium which will allow the small cells to pass.

It is common to all these methods that with more or less luck it is possible to bring about the preponderance of one or more groups of micro-organisms in a mixture, but it is obviously impossible to obtain in this way the exclusive presence of one particular species.

(*b*) **Dilution Methods.**—The second group of methods employed for physiological purposes embraces the dilution methods, or "fractional cultivation," the principle of which is to dilute the material to such a degree that it is ultimately possible to isolate a single cell. Brefeld used the dilution process for his botanical investigations of moulds, where he was able, owing to the size of the cells, to ensure that only a single cell was present in a small drop of *water* in the moist chamber Pasteur utilised *air* (*Études sur la bière*, 1876) as a diluting medium for preparing pure cultures He started from the fact that if nutritive liquids are exposed to the action of air, fermentation takes place, excited by the germs which fall on to the surface To isolate single germs from the yeast mass, he proceeded as follows —A small quantity of yeast was dried and ground with powdered gypsum. The fine dust was thrown into the air at as great a height as possible, and whilst the particles were floating down, a series of vacuum flasks were opened. Thus some of the yeast cells, which were finely distributed throughout the dust cloud, might penetrate singly into some of the flasks

The first application of the dilution method to bacteria was made by Lister (1878) To prepare pure cultures of lactic acid bacteria he first determined microscopically the number of bacteria in a minute drop of sour milk, counting them in several fields of the preparation, and thus calculating their whole number. He

then estimated the amount of sterilised water it was necessary to add so that after dilution there would be on an average less than one bacterium in each drop With five such drops he inoculated in one case five glasses containing boiled milk The result was that the milk in one of these coagulated, showing that it contained *Bacterium lactis*, whilst the four other glasses remained unaltered, and did not show the presence of bacteria The same method was subsequently employed by Nageli and Fitz (1882)

In comparison with the physiological methods the dilution method now described is a distinct advance, indeed we have thus approached much nearer to the goal On the other hand, it is clear that, even if the dilution is carried as far as in the case mentioned, in which only one of several flasks shows development, it is not yet proved that this one flask has received only one germ Thus, there is still great uncertainty, even in cases where the individuals with which we are working can be counted Moreover, it is extremely difficult to count individual bacteria, and often, indeed, quite impossible In all cases the accuracy of such calculations is very questionable Thus, the problem remains to be solved How are we to distinguish the flasks which have only received one cell from those which, notwithstanding calculation, have been infected with several cells?

In the case of yeast the process was further developed by Hansen (1881) He employed dilution with water, in the following manner:—The yeast developed in the flask is diluted to a given proportion with sterilised water, and after vigorous shaking, the number of cells in a small drop of the liquid is determined The counting, in this case, is easily carried out by transferring a drop to a cover-glass, in the middle of which some small squares are engraved, which form a starting point for the eye, and the cover-glass is then attached to a moist chamber (Fig 4), the drop must not be allowed to extend beyond the limits of the squares The cells present in the drop are then counted. Suppose, for instance, that 10 cells are found, a drop of similar size is transferred from the liquid, which must first be shaken vigorously, to a flask containing a known volume—*e g*, 20 c c of sterilised water This flask, then, will in all likelihood contain about 10 cells If it is now vigorously shaken for some time until the cells are equally distributed in the water, and 1 c c of the liquid introduced into each of 20 flasks containing nutritive liquid, then by calculation half of these 20 flasks should receive one cell each If the infected flask is strongly shaken and then allowed to stand, the single cells sink and remain on the bottom. It is evident that if a flask contains three cells, they will, in the great majority of cases, be separated by the vigorous shaking, and be deposited in three distinct places on the bottom. After some days, if the flask is raised carefully, it will be observed that one or more white specks have formed on the bottom of the

flask. If only one such speck is found, then in all probability the flask has only received a single cell.

It was by this method that Hansen prepared all his earliest pure cultures, with which he carried out his fundamental researches on alcoholic ferments

Solid nutrient media have also been employed for the preparation of pure cultures by the dilution method The foundation of such methods was laid by Schroeter (1872), who, in his researches on pigment-bacteria, employed slices of potato as a nutrient. He had observed that when such slices had been exposed for some time to the air, specks or drops of different form and colour made their appearance Each of these specks usually contained one species of micro-organism

Koch considerably developed and improved this method He at first prepared his pure cultures by means of streak infections in nutrient gelatine He afterwards devised a far better method, the plate-culture method (1883) The process is as follows :—A trace of the crude culture is transferred to a large quantity of sterilised water. From this a small quantity is transferred to a test-tube containing, for instance, a mixture of meat-broth and gelatine warmed to 30° C The tube is shaken in order to distribute the germs, and the contents poured on to a large glass plate, which is then covered with a bell-jar The gelatine quickly sets, and the germs are enclosed in the solid mass In a few days they develop to colonies—dots or specks which are visible to the naked eye The purity of the bacterial growths in the gelatine is ascertained, according to Koch, partly by their appearance.

An improvement in the method consists in the use of glass dishes with lids instead of glass plates, the Petri dishes (introduced by Salomonsen), into which the liquefied gelatine is poured, or the "roll-tubes" of Esmarch may be used, prepared by continuously rotating a test-tube round its longer axis until the inoculated gelatine has set in the tube, so that the whole of the inner surface is covered

When species are being developed which require a high temperature (at which gelatine would be liquefied), plates are made of agar, or of agar and gelatine The growth can be mixed with the liquefied material, or else spread over the surface of the soil, either by strokes of a platinum pencil, or by stabs with an inoculated needle

After selecting colonies, which appear to be pure, from a plate prepared in any one of these ways, a new plate-culture may be prepared from one colony. If all the colonies that develop on this plate are pure, it is probable that we are dealing with a pure culture.

When regarded more closely it will be seen, however, that there is no essential difference between the distribution of the

germs. in liquefied gelatine, and Lister's method of dilution by means of liquids The same uncertainty is always present, neither the macroscopical observation of the appearance of the colony nor the microscopical examination of its contents gives any surety of its only containing one species

The only possibility of securing a really pure culture in the gelatine consists in the direct observation of one individual germ and its development

Hansen did this for yeast by using Bottcher's moist chamber The lower side of the cover-glass is covered with a layer of wort-gelatine, in which the yeast cells are distributed. On account of the size of the latter, it is possible to see whether a single cell lies so wide apart from other cells that the colony developed from it will form a pure culture

The chamber is then either allowed to remain under the microscope, in order that the propagation of the germs may be directly followed, or the positions of well isolated germs are marked, either by dividing the glass-cover into small squares, or by means of the object marker, and the apparatus is placed in the incubator until

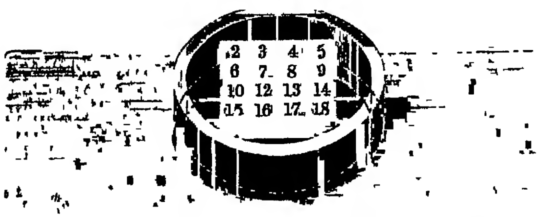


Fig 12 —Jorgensen's moist chamber with etched squares and numbers

the colonies are fully developed The cover-glass is then lifted off and placed under a bell-jar, so that the gelatine layer is turned upwards, and the colonies are transferred into flasks In the author's laboratory moist chambers like that represented in Fig 12 are used, the cover-glass being etched with 16 squares and numbers The situation of the cells is then marked on a sketch plan, which shows all the etched numbers and squares The author has altered the process by cementing the cover-glass on to the glass ring, and fastening the latter to the object glass with vaseline To remove the chamber, the ring is lifted off, and this is a more convenient and more certain process than lifting the cover-glass, for it is possible to transfer the colonies without inverting it. On one cover-glass there may be 50 to 60 well isolated germs When the colonies are conveyed to the flask by means of a small piece of platinum or copper wire, which has been previously ignited and cooled, the culture is momentarily in the air, and is then exposed to contamination But the danger of contamination at this, the single weak point, is reduced to an insignificant minimum,

and disappears if the operation is performed in a small enclosed sterile space, for instance, in a small cupboard with glass sides sufficiently large to admit the apparatus and the operator's hands In this way the transference of the colonies is effected with all possible security From the first flask the culture can be transferred without contamination to a continually increasing number of larger flasks.

For the pure cultivation of brewery, distillery, and wine yeasts, vigorous cells must be conveyed to the gelatine in the moist chamber According to J C Holm, on an average only about 4 per cent. of the inoculated cells do not develop, whilst from a growth of yeast that is taken at the end of the fermentation, in which the cells are weakened, about 25 per cent do not develop. It is usually preferable to convey a small average sample of the yeast into wort or must, and then to use the very young growth, which is developed when the first trace of fermentation is observed. To decide whether any of the selected yeasts are of value for industrial purposes, a large number of cells must be isolated, as indicated by the author as early as 1885 After years of experience, it has proved impossible to speak of a preponderant species or race from which any individual can be chosen The single type or species contains within itself so many varieties which have come to development under the conditions existing in practice, that a careful choice must be made from these A thorough study of the different varieties of the type by means of comparative experiments will show which of the cultures is of the greatest value in practice

As early as 1883, Koch's method of plate-culture was tested by Hansen. He prepared a mixture of two species of yeast which can be distinguished from each other microscopically—viz, *Saccharomyces apiculatus*, and a species of the group *S cerevisiæ*. This mixture was introduced into wort-gelatine, and after shaking was poured on to a glass plate Of the specks formed, about one-half contained one species exclusively, the other half the other species, and in one of the specks both species were found.

A similar control was carried out for bacteria by Miquel (1888), who introduced 100 colonies from a plate-culture obtained in an air analysis into 100 flasks containing meat-broth with peptone. The examination of the growths developed in the flasks showed that they contained 134 different species of micro-organisms. This evidently depends upon the fact that it is very difficult, and often quite impossible, to separate all germs of bacteria and other organisms from each other by shaking the gelatine mixture

Holm has subjected the method to a thorough analysis (1891), in the case of a large number of yeast species, absolutely pure cultures of which were prepared by the Hansen method. The result of 23 series of experiments with different mixtures was that only in a single case were 100 colonies developed from 100 cells In all

the other series the method proved faulty. In the most unfavourable case 100 colonies were formed from 135 cells, and the average number obtained was 100 colonies from 108 cells This proves the plate method to be defective also in the case of yeast

A modification of Brefeld's culture of a single cell in a hanging drop is that known as the drop culture, introduced by P. Lindner in 1893 It consists in conveying to a cover-glass a number of small drops of a diluted culture in a nutritive liquid by means of a mapping pen The cover-glass is fastened by a ring of vaseline on to a hollow-ground object glass, and those drops are noted that contain only one cell Care must, therefore, be taken that the drops do not flow together before the pure culture is conveyed to a flask.

Burri attempted to solve the problem of preparing pure cultures of bacteria under direct observation of single cells by the help of his Indian ink point culture He dilutes ordinary liquid Indian ink with water in the proportion of 1 to 10, and after sterilisation infects with the bacteria, and then dilutes to such an extent that small drops of 0 1 to 0 2 mm. diameter contain on an average a single germ Such drops are placed at suitable distances with a mapping pen on the surface of a layer of nutrient gelatine, where they immediately evaporate, and are then protected by flamed cover-glasses Much smaller drops can be deposited on gelatine than on a cover-glass The contents of these specks can then be controlled under a high power, and those noted which contain a single germ, a process that is rendered easier by the fact that the bacteria appear clear on a greyish-brown ground. If they can grow in gelatine the development is allowed to continue, but if they require high temperatures they may be conveyed to an agar plate by cautiously raising the cover-glass, and as the speck of ink is more firmly fastened to the glass than to the gelatine, the germ is carried with the glass, so that it can be conveyed along with this to the agar plate After removing the germ from the gelatine plate a drop of nutritive liquid can be placed on the ink fleck of the cover-glass, and so the germ may be developed in a liquid The process can also be used for the cultivation of anaerobic bacteria

Anaerobic bacteria demand special methods of cultivation, in which the atmospheric oxygen must be removed both from the substratum and from the space in which the bacteria are growing Pure cultivations may be carried out in nutrient gelatine or agar with 1 or 2 per cent dextrose, in tubes filled almost to the top, the bacteria growing in the bottom layers.

A still better process consists in removing the air from the test-tubes by means of an air pump, whilst the glass is immersed in water at 30° to 35° C , after which it is hermetically sealed. Another method is to remove the air with a current of hydrogen This is conveniently carried out in the following way (*Frankel*) :—A wide test-tube is fitted with an india-rubber stopper with two holes

carrying two glass tubes, one of which reaches to the bottom of the test-tube; the other terminates just below the stopper When the vessel has been covered with nutrient gelatine or the like, and sterilised, it is inoculated with the growth, and a stream of hydrogen is passed through the long tube The tube is sealed up as soon as the air is completely driven out The stopper is sealed with melted guttapercha, and if gelatine or agar is being used the test-tube is rotated round its longer axis until the material has solidified The bacteria develop slowly on the inner surface of the glass

The cultivation can also be carried out by utilising a substance that will absorb oxygen—*e.g*, pyrogallic acid (1 gram of the dry reagent in 10 c c of a one-tenth normal potash solution). To carry this out the open test-tube or plate-culture is placed in a larger air-tight test-tube or vessel containing the reagent The nutrient liquid may also be sterilised at the bottom of a test-tube, into which a stopper of hygrophobic cotton-wool has been introduced, above the latter is placed a stopper of hygroscopic cotton-wool, thoroughly moistened with the alkaline pyrogallol solution, and the tube is then closed with a rubber stopper An example of the velocity with which oxygen is absorbed is cited by Riemsdijk: 400 c c. of air at rest may in thirty minutes be deprived of its oxygen by 10 c c of KOH (20 per cent), and 3 c.c pyrogallol (44 per cent). A rise of temperature will increase the rapidity of oxygen absorption

The culture may also be covered with paraffin, vaseline, oil, plates of mica, etc

Reducing substances like grape sugar, especially in an alkaline solution, or minute quantities of formic acid or sodium indigo sulphonate may be added to the nutritive substance, in order to favour the growth of anaerobes

If it is wished to ascertain with certainty, in using one of these processes, when all oxygen has disappeared, a concentrated alcoholic solution of methylene blue may be used as an indicator, as soon as the last trace of oxygen is absorbed or removed the indicator will be entirely decolourised The same applies to the addition of indigo carmine (neutral sodium indigo sulphonate).

8. Counting the Yeast Cells.

The multiplying capacity of the yeast cells can be estimated by directly counting the cells that are present in a given volume of the liquid at different stages of the fermentation

The counting is performed by means of an apparatus constructed by Hayem and Nachet, and by C Zeiss (Fig 13), which was first employed for counting the corpuscles of blood (hence termed *hæmatimeter*) The hæmatimeter consists, as shown in the diagram, of an object glass on which a cover-glass of known

thickness (*e g.*, 0·2 mm.) is cemented, and from the centre of which a disc has been cut out A small drop of the liquid containing the cells is brought into the cavity thus formed, a second cover-glass is placed over the opening, and thus rests on the cemented and perforated cover-glass The drop of liquid must not be so large that the pressure of the cover-glass causes it to flow out from the enclosed space, yet it must be high enough to be in contact with the cover-glass The thickness of the layer of liquid is then known. In order to determine the other two dimensions, and thus be able to work with a given volume of liquid, one of the well-known forms of micrometer is introduced into the eye-piece of the microscope It may consist of a thin piece of glass on which 16 small squares are engraved The actual value of each of these squares is known when a given system of lenses is employed, and thus, when the square is projected on the object, a small prism of known volume is defined In certain cases it may be more expedient to make use of an appliance constructed by Zeiss, of Jena, from the instructions of Thoma, which consists of a fine system of squares of known size, engraved on the object-glass itself at the bottom of the cavity.

Fig 13—Hæmatimeter—*a*, object-glass, *b*, cemented cover-glass with circular opening; *c*, cover-glass

This also improves the microscopical definition of the cells which are on the bottom of the chamber

When it is merely desired to determine the rapidity with which the cells multiply by repeated observations of the number of cells in the same volume, it is quite superfluous to determine its size, it is simply necessary to work always with the same volume

The sample taken should always be a fair average. In most cases it must be diluted and thoroughly agitated for a long time, in order to obtain an equal distribution of the cells, the specific gravity of the liquid must also be such that it will allow the cells to remain suspended in it for a short time A small drop is then withdrawn in a capillary tube, transferred to the counting apparatus, and covered with the cover-glass. The apparatus is allowed to remain at rest for some time, in order that the cells may settle to the bottom of the enclosed space, and on this account the specific gravity of the liquid must not be greater than will allow this to take place in a convenient time Both these requirements are generally satisfied by the wort employed in breweries.

If it is found that the determinate volume contains too many cells to be counted with certainty, the liquid must be diluted. This may be advisable for other reasons, partly to prevent the formation of froth, which may otherwise form abundantly from the violent agitation, and partly to isolate the single cells which frequently cluster as colonies in the wort, and are not always separated by shaking. Finally, it is necessary, whilst the counting is going on, to arrest the development of the yeast cells in the sample.

Hansen found that dilute sulphuric acid (1 to 10) on the whole answers these requirements, hydrochloric acid, ammonia and caustic soda may also be used, but they are not so good. If very great dilution is required, distilled water may be added, after the addition of one to two volumes of dilute sulphuric acid.

When the different volumes of liquid are measured with accuracy, and particular care taken that the cells are thoroughly distributed by vigorous and prolonged shaking, the determination can be made with great accuracy. Two similar dilutions must always be made, and samples taken from each for counting As a matter of course, experiments must also be made to determine the number of the small squares, the cell contents of which must be counted to arrive at a true average Such counting and determination of the average numbers is continued until the number finally obtained is found to have no further influence on the average value The number of countings necessary, and the accuracy generally, depends on the experience and care of the observer

CHAPTER II.

BIOLOGICAL EXAMINATION OF AIR AND WATER.

THE investigations into spontaneous generation already referred to naturally led to the study of the organisms in air, and after Pasteur, in particular, had demonstrated that air contained not bacteria only, but also fungi giving rise to alcoholic fermentation, air analyses acquired an interest for the zymophysiologist, and for the fermentation industry Such comprehensive researches are now available that it has been possible to arrive at an idea of the biological composition of the air on a large scale, and to form a judgment of these conditions in relation to the brewing industry. At first, when it became known that crowds of living germs, capable of development, could occur even in very small volumes of air, there was a natural inclination to exaggerate their effect in practice, and to attribute any excessive growth of disease germs in a fermentation to the direct influence of the air

An exhaustive study of the conditions occurring in practice, carried out in recent years under systematic biological control, has shown that this influence had been exaggerated, and that it is possible, even where an air analysis has shown the presence of numerous germs capable of producing disease in a fermenting liquid, to suppress the partly dried and weakened germs falling into the liquor by the addition of the excessive number of yeast cells contained in the pitching yeast

Large growths of disease-producing organisms were only found in practice if they had been allowed to develop on certain infected areas. The germs in the air are thus only indirectly the cause of disturbances in practice, and under normal conditions can seldom be of importance

The majority of **air analyses** have been undertaken with a view of throwing light on the obscurity which surrounds most contagious diseases, nearly all of which are, as is well known, attributable to the agency of micro-organisms. With regard to the organisms of fermentation, these have been investigated by Pasteur, and, later, especially by Hansen. The French *savant* stated that, whilst these germs are always floating about in the air, *they are present in much larger quantities in the dust which settles on the vessels and apparatus employed* The actual fungi giving rise to alcoholic fermentation are present in comparatively small numbers in the

air, whilst the germs of moulds are more frequent, he further showed, as was subsequently done by Tyndall, that the germ-contents of the air vary both with regard to quantity and species. These results were obtained by exposing beer-wort, wine-must, or yeast-water containing sugar, in open, shallow dishes, at different places, and examining their contents after some time for microscopical germs Pasteur also employed for this purpose the so-called vacuum flasks, containing nutritive liquids and rarefied air. On opening the flask a sample of germ-laden air could be drawn in

The most important air analyses undertaken in recent years are, without doubt, those carried out by Miquel, the director of the laboratory specially arranged for this purpose at Montsouris, near Paris. His fellow-worker, Freudenreich, has also made valuable contributions to our knowledge of this subject

Miquel performed his first experiments with a so-called Aeroscope (Fig 14), which is constructed in the following manner:-

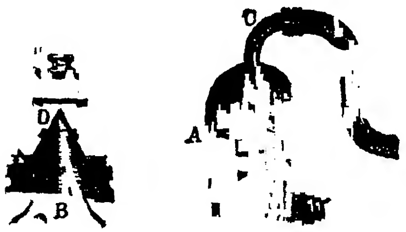


Fig 14 —Aeroscope

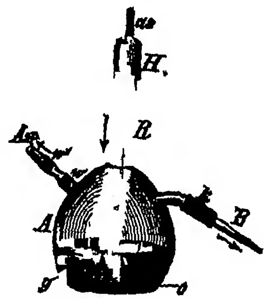


Fig 15 —Miquel's apparatus for air analysis

A bell-shaped vessel, A, is provided with a tube, C, through which air can be aspirated A hollow cone, shown in the left-hand figure, is screwed into the bottom of A, the mouth of the cone, B, points downwards, in the apex, D, of this cone there is a very fine opening through which the air is aspirated, and immediately over this opening is fixed a thin glass plate covered with a *mixture* of glycerine and glucose The particles carried in by the air settle *to some* extent on the viscous mixture. The intercepted micro-organisms are distributed as equally as possible on the glass plate, and counted under the microscope This method is defective in so far as it gives no information on the most *important* point—namely, which and how many of the intercepted germs are actually capable of development

In order to determine the number of germs capable of development, and also their nature, Miquel employs the following apparatus (Fig 15) —The flask *A* has fused into it a tube, *R*, tapering below and nearly reaching to the bottom, the upper end of this

is fitted with a ground cap, *H*, provided with a narrow filter-tube containing sterilised cotton-wool, asbestos, or glass-wool, *us* On one side of the flask is a tube, *Asp*, which is constricted in the middle, and is provided with two cotton-wool plugs, *w′* and *w* On the other side is another glass tube connected by rubber, *k*, with the tube *B*, which is drawn out to a point, and closed by fusing the end. The flask is partly filled with distilled water, and the whole apparatus sterilised. When the apparatus is to be used, the tube *Asp* is connected with an aspirator (*e g*, a bottle filled with water and provided with an outlet cock), the cap *H* is taken off, and the air then passes, bubble by bubble, through the opening *o*, through the water *g*, and out through the cotton-wool plugs of the tube *Asp* As all the germs in the air are not retained by the water when the air-bubbles ascend through the latter, the cotton-wool plug *w* is intended to catch those which get past the water When the experiment is finished, the cap *H* is replaced over the tube *R*. By blowing through *Asp*, the liquid is made to ascend in *R*, in order that any germs which may have settled on the walls of the tube may be washed down into the liquid Then, by blowing with greater force, the inner cotton-wool plug *w* is driven down into the liquid, and its germs shaken off into the latter After sterilising the thin tube *B* in a flame, the point is nipped off, and the liquid is now—by blowing through *Asp*—transferred, drop by drop, into a large number of flasks containing sterilised broth

The main object then is, by means of preparatory experiments, to obtain such a dilution of the air-infected water that a considerable proportion of the small flasks (one-half for example) remain sterile after inoculation, or several samples of the water may be diluted to different degrees, and a series of flasks inoculated from each dilution ("fractional cultivation") If a large number of the flasks show no development of organisms, there is a certain probability that in each of the remaining flasks in which growths have developed, only one germ has been sown A simple calculation will then show how many germs capable of development in the medium employed were present in the volume of air aspirated through the original flask

By these methods of investigation Miquel found that similar volumes of air in the same locality contained at different times a varying number of bacteria Continued rain purifies the air from bacteria to a marked extent, and their number continually diminishes as long as the earth is moist, but when the ground dries, it gradually increases again Thus in the dry seasons of the year the number of bacteria is usually the greatest, whilst the moulds, which thrive best in moisture, and carry spore-bearing hyphæ, which project upwards, are most abundant during the wet seasons The purest air is found in the winter time, the air of towns is less pure than that of the country; germ-free, or nearly germ-free air is found

at sea and on high mountains. In certain places—hospitals, for instance—the air has been found to be very rich in bacteria; in one case even fifty times richer than the air in the garden at Montsouris.

An entirely different method for determining the organisms contained in air is that employed in Koch's laboratory, and more completely developed by Hesse. A glass tube, about 1 metre long and 4 to 5 cm. wide, is closed at one end with a perforated india-rubber membrane, over which another non-perforated cap is bound. A little liquefied nutrient gelatine is then poured into the tube, after which the other end is closed with an india-rubber stopper, through which passes a glass tube plugged with cotton-wool. The whole apparatus is then heated sufficiently to render it sterile, after which the tube is placed in a horizontal position, so that the gelatine sets in a layer in its lower part. When the air is to be examined, the outer india-rubber cap is removed and air slowly drawn through the tube. The germs contained in the air settle down on the gelatine, and after the aspiration is concluded the tube is again closed and placed in the incubator, where some of the germs produce visible colonies, which are easily counted. The results show that with a sufficiently slow current of air, the bacteria, which are often floating about in the air in larger or smaller aggregations (frequently clinging to dust-particles, small fibres, or splinters), settle sooner than the mould-spores; so that the gelatine in the fore part of the tube generally showed a preponderance of the bacteria colonies, whilst the mould-spores developed further on.

Miquel's method is to allow the air to pass through a hollow cylinder of solidified gelatine, in which the germs are retained.

Hueppe, v. Schlen and others use liquid gelatine for air analyses, the air being aspirated through the gelatine, after which the latter is poured on to glass plates.

Frankland, Miquel, Petri, and Ficker use porous solid substances for the filtration of air for analytical purposes; as, for example, powdered glass, glass-wool, sand, sugar, etc. The sand-filter employed by Petri is 3 cm. long and 1·8 cm. wide. It is packed with sand, previously ignited, the size of the grains being from 0·25 to 0·5 mm. Two such sand filters are placed one behind the other in a glass tube. In the first filter all the dust-particles containing germs should be retained, whilst the second filter serves as a control. The sand charged with germs is distributed in shallow glass dishes and covered with liquid gelatine. The germs accompanying the dust-particles will then form colonies in the gelatine.

When samples of the air contents are to be sent from one place to another, these air filters will answer the purpose. On receipt of a sample, the sand may be washed into gelatine or, preferably, into sterilised water. After vigorously agitating the water, it is

added in drops to flasks containing nutritive liquid, or it may be used in plate-cultures

When samples of air are to be sent to the author's laboratory, short cylindrical glasses are used, having india-rubber stoppers, which project well beyond the mouth of the glass The latter are half-filled with sterilised water or with the nutritive liquid in actual use. When the glass is opened at its destination, the stopper is placed by it with the wet end turned upwards, care being taken, of course, that this is not touched A suitable time having elapsed, the stopper is replaced and tied down.

Miquel has raised an objection to the employment of gelatine plates for this purpose, based upon numerous experiments He asserts that many bacteria, when exposed to a temperature of 20° to 22° C., require a fortnight's incubation before developing distinct colonies in gelatine, on the other hand, there are species which very soon liquefy the gelatine, thus rendering further observation impossible The same is the case with the moulds, which often spread all over the plate in a few days Thus, it becomes necessary to count the colonies at so early a stage that many of them are not yet visible An additional drawback to the gelatine plates is, that the development cannot take place at a temperature higher than 23° to 24° C, otherwise the gelatine will liquefy, but many species of bacteria present a characteristic development only at considerably higher temperatures Other species, moreover, do not develop in gelatine at all, but only in liquids. Finally, it is urged as a very material objection to the gelatine plates that many of the colonies consist of several species Miquel proved this by introducing the colonies, one by one, into meat decoction with peptone, and then again preparing plates from these growths. This is in part due to the fact that the bacteria, as shown by Petri, often occur in aggregates in the air, and these will either fall directly on to the gelatine plate or become mixed in the liquid gelatine, where it would be very difficult to separate the individuals from each other by agitation

E C Hansen's investigations of the air were made between 1878 and 1882 His main object was to throw light on questions affecting the fermentation industries. As is well known, his researches on *Saccharomyces apiculatus* (1880) were partly based on work of this nature Since the question concerned the organisms which occur in brewing operations, the choice of a nutritive liquid was easily determined—namely, wort as ordinarily employed in breweries. The apparatus used consisted either of Erlenmeyer flasks closed with several layers of sterilised filter paper, the contents of which were boiled for a certain time, or of vessels similar to Pasteur's vacuum flasks, the necks of which were drawn out to a fine point, and closed with sealing-wax while the contents were boiling. A little below the point a scratch was made with a file, so

that the point might be easily broken off when it was desired to admit air.

When these flasks had been filled with the air of the locality to be examined, they were again closed with sealing-wax and thoroughly shaken in order to mix the contents of the infiltrated air with the liquid. The flasks were then put aside for a longer or shorter time, lasting in some cases for six weeks, and their contents examined under the microscope

In these investigations Hansen often found that the wort remained bright and apparently unchanged, even although a growth had taken place Hence the examination with the naked eye alone cannot be relied on He names the following forms which, when present in a feeble state of growth, cannot be detected macroscopically —*Aspergillus*, *Mucor*, *Penicillium*, *Cladosporium*, *Bacterium aceti* and *Pasteurianum*, and *Mycoderma cerevisiæ*. Even when these micro-organisms have formed vigorous growths, the wort used has remained bright

It was further shown that pure cultures may often be obtained by the use of these flasks, when only one species gained access to the flask along with the air It very seldom happened that three or four species were found in the same vessel. This arises from the fact that only a very small volume of air enters each flask. The advantages of this are evident A true knowledge of these germs can only be obtained when they have developed, in cases where several germs penetrate into the same flask, the strongest germ would by its growth, in all probability, prevent the development of the others, so that these would not be detected in a subsequent examination. At the same time this method necessitates the opening of a large number of flasks, which makes the operation cumbersome and costly. As the flasks only show what was present in the air at the moment of opening, Erlenmeyer flasks were also used to give supplementary information, for which purpose they were allowed to remain in the same locality for a long time, in some cases as long as 48 hours

After these preliminary remarks, we will give a brief summary of the results obtained by Hansen.

He confirmed the statement made by Pasteur and Miquel that the air at adjacent places, and at the same time, may contain different numbers and different species of organisms; and he found that this holds good for adjacent parts of one garden Hansen mentions, amongst other factors determining the distribution of micro-organisms, that those forms, for instance, which in the first half of July commonly occurred under the cherry trees in the garden, were in the latter half of the same month entirely absent from this locality, further, that organisms which at one time could be found under the cherry trees, but not under the vines, were to be found later only under the latter As a proof of the

inequality of distribution of the organisms, he showed that flasks opened in the same place in the same series of experiments often had the most diverse contents.

The experiments with vacuum flasks have further taught us that the micro-organisms of the air often occur in groups or clouds, with intermediate spaces, which are either germ-free or only contain a few isolated germs As the organisms are not generated in the air, but on the earth and on fruit, it follows that their presence in the air must be dependent on the condition of the surface of the ground and of the fruit, which again depends to come extent on the weather

Hansen's numerous analyses have further proved that the *Saccharomycetes* comparatively seldom occur in the dust of the air Their number in the open air increases from June to August to such an extent that flasks at the end of August and the beginning of September are frequently infected with these organisms, after which a decrease takes place The *Saccharomycetes* which are found at other times of the year in the atmosphere may be regarded as unimportant in numbers and accidental in occurrence As most species of the *Saccharomycetes* have—like *Saccharomyces apiculatus* —their winter quarters in the earth, and their propagation areas on sweet succulent fruit, the latter must be considered as the most important source of contamination During the same season bacteria are also found in the largest numbers This constitutes a real danger in technical operations, since wort, when spread in a thin layer on the open coolers, is exposed to a source of contamination from the atmospheric germs

Bacteria are found in the flasks in somewhat greater number than the *Saccharomycetes*, whilst the moulds occur in still greater numbers Amongst the latter *Cladosporium* and *Dematium* are especially prevalent in gardens, and after these *Penicillium*, whilst *Botrytis*, *Mucor*, and *Oidium* occur less frequently

After Hansen had thus demonstrated which of the micro-organisms existing in the open air are capable of developing in flasks with sterilised wort, he proceeded to communicate the results of his examination of different parts of the brewery.

When grains (draff) are allowed to stand in the open air, they evolve, as is well known, acid vapours; and since they always harbour a rich growth of bacteria when they remain exposed for a short time, the question naturally presents itself, what is the condition of the air in the neighbourhood of heaps of grain? It was found that only 30 per cent of the flasks opened in these areas became infected, and of these 3 6 per cent with *Saccharomycetes* and 2·4 per cent with bacteria, whilst parallel experiments in the garden gave a contamination of about 44 per cent, of which 8 5 per cent were bacteria The air near the grains thus contained fewer bacteria than the air in the garden. The most abundant

contamination was that of moulds, as in all other localities. After a thorough examination Hansen came to the conclusion that, without doubt, scarcely a single organism which entered the flasks proceeded from the grains The result tends to show that in this, as in other cases, air does not take up any essential organisms from moist surfaces.

This, however, must not be misunderstood to mean that grains may be allowed to accumulate, without risk, and that after removal, the residue may be exposed to the weather. It is clear that this would constitute a great danger. When the remains become dry and are blown about in the air as dust, masses of bacterial germs will be carried up at the same time, and will, without doubt, constitute a source of frequent bacterial contamination For this reason, places where grains have remained for any length of time must be washed with limewater or, preferably, with chloride of lime *

In a corridor leading to a room where barley was turned, the flasks always showed greater contamination than anywhere else; bacteria especially were found in them in great abundance

On the malt floors the condition of the air was also characteristic; it always contained a very strong contingent of mould spores In the case in question these consisted of *Eurotium aspergillus*, which was otherwise rare On the malt itself, as usual, *Penicillium glaucum* occurred most frequently

The greater interest, however, attaches to the examination of the different fermenting-rooms, partly in the "Old" Carlsberg brewery and partly in the brewery "N" In the former the air contained fewer organisms than in any of the rooms examined during the whole research, in the fermenting-cellars of the brewery "N," on the contrary, a large number of flasks (55, 75 to 100 per cent) were infected. The organisms which occurred in the air of these cellars were—*Saccharomyces cerevisiæ*, *Mycoderma cerevisiæ*, *S. Pastorianus*, *S ellipsoideus*, *Torula*, and other yeast-like cells; further *Penicillium*, *Dematium*, *Cladosporium*, and rod bacteria. Hansen was thus enabled, by a favourable chance, to contrast the state of the air in the most important part of these two breweries; on the one hand an almost germ-free air, on the other hand an atmosphere teeming with germs That the product of the latter place must have been affected by the atmospheric conditions then existing admits of no doubt, and this brings us face to face with one of the most important of all facts to the practical brewer—*i.e.*, that the air in the fermenting room itself may contain a multitude of those germs which are productive of the most calamitous

* The germs are not killed during the treatment of the grain in drying machines Such forms of apparatus, therefore, constitute a very great danger in the brewery, since dust-clouds of bacteria may be transported from the dried grains to the open coolers or into the fermentation vessels

results It is, however, possible to keep the air free from these invisible organisms, and there is no doubt that the results recorded are directly due, first, to the purification of the air entering the fermenting-room by passing it over brine, and, secondly, to the rigidly maintained order and cleanliness in the cellars of the Old Carlsberg brewery. Hansen's investigations, therefore, point a moral which cannot be too frequently emphasised.

Saito carried out very comprehensive investigations on the distribution of moulds at different times of the year in many places in Tokio, both in the open and indoors, with the aid of Soja-gelatine (Soja, decoction of onions and cane sugar), confirming by this means the results of earlier investigations already cited

More recent determinations in the same localities of the distribution of aerobic *bacteria* led to the same results as those arrived at by earlier workers

Of particular interest are the observations subsequently made by Saito on the *yeasts* occurring in the air at Dairen, Manchuria. His tabulated data proved that yeasts react like bacteria to the degree of humidity of the air, as opposed to moulds. A wide investigation proved, for all three groups of organisms, that the number of germs in the air is dependent on a combination of meteorological conditions—temperature, rainfall, humidity, and wind. As for the number of yeast germs in the air, the author, contrary to all expectation, found it to be less in the warmer months of the year (March to August) than it was in the colder months (September to February)

In the three series of air analyses referred to, Saito met with a number of species which appear to be indigenous to those countries. For further particulars, see the systematic part of this book

The life-history of the yeasts is described in the chapter on the Biological Relationships of Yeast

The zymotechnical analysis of **water** has been of greater value to the brewing industries than the analysis of air The germs contained in water which give rise to disease in fermentations and fermentation products are not usually so enfeebled as those in air, and water on many occasions comes into closer touch with the different products during manufacture than does air. The examination of water in reservoirs, and the effect of filtration on the micro-organisms, is of especial practical value.

A few details may be quoted here from the researches of Holm and of the author as examples of the results obtained by such investigations in the fermentation industry

Holm's researches showed that among the various micro-organisms in water the *moulds* are those which develop most quickly in flasks containing wort and beer, and generally also those which occur in largest numbers in the flasks *Penicillium glaucum* and *Mucor stolonifer* were found among them.

Next to the moulds come the bacteria when wort is infected with water, whilst if sterilised beer is used, they develop only scantily. The following bacterial forms were found :—*Bacterium aceti*, *Bact. Pastorianum*, a third form which made the beer slimy and ropy; and lastly, several species which imparted a disgusting smell to the wort.

Yeast-like cells were of rare occurrence. Holm did not observe any growth of *Saccharomycetes*, although some *Torula* forms and *Mycoderma* occurred.

The number of these germs varied at different times of the year, yet it did not seem to be dependent on the season—the rainfall, the condition of the surface water, and of the air had great influence. Of practical importance was the discovery of strong contaminations, injurious to wort and beer, in reservoirs situated near granaries and malt-lofts insufficiently protected against dust. It was also shown that water which had been filtered through charcoal filters contained much larger numbers of wort bacteria than the unfiltered water.

The water analyses made in the author's laboratory during a period of more than twenty years have given the following chief results :—The samples of water in only very few cases were found to contain *Saccharomycetes* (culture yeasts or wild yeasts). In one series of analyses *S. anomalus* and *S. membranæfaciens* were met with. The bacteria observed by Holm which produced slime formation or imparted a putrid smell to the wort, occurred very frequently. If a pure yeast was infected with such species and used for pitching hopped wort, these bacteria did not usually develop further. Although, however, the bacteria did not develop during the fermentation, a difference was often observable between the condition of the beer and that of beer fermented with pure yeast. Acetic acid bacteria were not infrequently found in the analyses, and were usually able to assert themselves in the flasks, even in competition with rival species. In a few cases the experiments with wort showed a growth, and sometimes even an abundant one, of *Sarcina* forms, which did not occur in the parallel series of experiments with sterile beer. They rendered the wort turbid, and imparted a peculiar smell to it. Among the moulds the following were the most frequent :—*Aspergillus*, *Mucor stolonifer*, *M. mucedo*, *Oidium lactis* and *Dematium*-like forms. In the water conduits of the breweries a coherent layer of *Crenothrix* was not infrequently found.

In many cases it has been proved that water received a very considerable contingent of its wort and beer organisms in the reservoirs or conduits, and it may safely be asserted, as the result of many years' experience, that brewery water is most seriously contaminated in the brewery itself.

Biological analyses of natural and artificial ice have shown

that in both, organisms can exist capable of developing in wort and beer. *Sarcina*-like bacteria can also be introduced along with ice into these liquids, and may develop freely in them In artificial ice, the inner snowy layer of the ice-block appears to be particularly rich in micro-organisms

If large quantities of water are to be analysed, it is of the utmost importance to take due care that real average samples are obtained

Hansen gives the following method for the zymotechnical analysis of air and water, a method based upon a long series of comparative trials.

The principle underlying it is as follows —For brewing purposes it is only necessary to know whether the water and the air contain germs capable of developing in wort and beer This cannot, as was formerly assumed, be ascertained by means of the meat decoction peptone gelatine employed in hygienic air and water analysis The zymotechnologist has this great advantage over the hygienist, that he is in a position to make direct experiments with the same kind of liquid as that employed in practice—namely, wort All disease germs that have hitherto been shown with certainty to occur in beer are also capable of developing in wort. Hansen's comparative investigations have proved that the use of gelatines introduces great sources of error Thus, for instance, in a series of comparative experiments with corresponding samples of water, the following numbers were obtained —In Koch's nutrient gelatine —100, 222, 1000, 750, and 1500 growths obtained from 1 c c of water, in wort—0, 0, 6 6, 3, and 9 growths, whereas, in beer, none of these water samples gave any growth. In another series, Koch's gelatine gave for 1 c c of water 222 growths, wort gelatine 30, but none of the flasks containing wort or beer, after infection with the water, showed any development of organisms Thus, only very few of the great number of germs living in the water developed in wort or beer

Hansen has further shown that in zymotechnical analyses of water and air, it is a mistake to employ gelatine at the outset, and then to transfer the colonies that have been formed into wort Thus, he demonstrated by experiment that several of the bacterial germs occurring in atmospheric dust and in water are capable of developing in nutrient gelatine, but not in wort, whilst several of these species become invigorated to such a degree, after having formed a new growth in the gelatine, that they are then enabled to develop in the less favourable medium, hopped wort Another, and a still greater, objection to the gelatine method is that several organisms, and just those of importance, do not develop at all when transferred directly to the gelatine in the enfeebled condition in which they generally occur in atmospheric dust and in water

Temperature naturally plays a much more important part in the development of the germs on gelatine plates than in nutritive

liquid, at a less favourable temperature they will develop with greater difficulty in gelatine, owing to its deficiency in nutriment

Reference may also be made in this connection to the difficulties that are encountered in determining the number of germs which can develop on gelatine plates Species of frequent occurrence in water, that tend to liquefy gelatine, generally develop very rapidly, and may encroach so extensively on the space that other species do not succeed in forming colonies at all, others require so long an incubation before visible colonies are formed that the examination of the plates is often concluded before these growths appear.*

Based upon these observations, Hansen devised the following method.—Small quantities of the water, either in its original state or diluted, are added to a series of Freudenreich flasks containing either sterilised wort or beer † After incubation at 25° C. for fourteen days the contents of the culture flasks are submitted to examination If only a part of them show any growth, the rest remaining sterile, it may be assumed with some certainty that each of the flasks belonging to the former set has received only a single germ Information is thus gained concerning the number of germs capable of development existing in an ascertained volume, and the different germs are also under more favourable conditions for their free development An exact examination will then show to what species these germs belong

Although wort-cultures give a very small number of growths by this method in comparison with plate-cultures, yet in many cases the number of wort-growths will still be too high, for these growths are able to develop in the flasks undisturbed and without hindrance from other organisms, but when wort is mixed with good culture-yeast in the fermenting vessel, many of these germs will be checked. Further, the flasks which show a formation of mould will have no importance for the brewery itself, but only for the malthouse. In order that the conclusions based on the results should approximate more closely to practical requirements, Hansen proposes the following method of procedure—The flasks containing a development of yeasts and bacteria are divided into two groups—(1) those in which the growths appear rapidly, and (2) the remainder, in which they make their appearance later, for instance, after five days Among the latter are those species which develop less readily in wort To these, then, less importance is attached in forming an opinion as to the nature of the water or air.

The same principle is used by Wichmann, who endeavoured to give a numerical expression for the "harmfulness" of water—that is to say, to express to a definite degree the destructive property

* In comparative investigations, as, for instance, the examination of air and water before and after filtration, gelatine plates are usually employed, the possible sources of error accompanying their use must, therefore, be borne in mind

† In the analyses of air the germs are aspirated into sterilised water, or first into cotton-wool and then transferred to water

of water with regard to wort and beer He impregnated a series of Freudenreich flasks, containing sterile, clear wort or beer, with different volumes of water, and noted the day on which a change in the contents of each flask (cloudiness, formation of a skin, fermentation) became visible The more rapidly decomposition sets in, and the smaller the necessary quantity of water, the more noxious should be the character of the water. By expressing numerically the time (setting in of decomposition), and the quantity of water, the figure specifying the destructive capacity will be a product of the two factors Beer, however, possesses a greater power of resistance than wort, so that in cultivations in the former, the coefficient must be correspondingly reduced

Lindner added sterile wort to the water, and distributed the mixture by means of a pipette drop by drop into a series of Petri propagating dishes The growths which developed were counted, and from this the number of germs per c c was calculated.

The results of many years' experimenting in the author's laboratory have led to conclusions which are at variance with those stated above The first growths to appear in the wort flasks are almost always putrefactive bacteria, water bacteria, and the like; just those forms which are of little interest in brewing operations, because they do not develop in the finished product The special technical character of the analysis is lost if the time taken for the appearance of the bacteria is made the basis on which the character of the water is judged Moreover, in forming an opinion as to the character of water from the zymotechnical standpoint, it is not essential that the quantity of wort-bacteria in a given volume should be estimated. On the contrary, when growth in the flasks has been allowed to continue for some time, it has been shown that in more advanced stages species appear which are known as disease germs, as, for instance, the wild yeast. Thus it is the last stage of the development in the flasks which is of real importance In making the investigation, both the wort and the beer flasks should be infected with small quantities of water In some flasks the water is added in an undiluted condition, in others it is more or less diluted Samples are taken from time to time for microscopical, and eventually, for microbiological examination, in order that the micro-organisms which appear may be more closely investigated in regard to their action on wort and beer. For water analyses in connection with distilleries and allied industries, sweet wort is usually employed. This has been found to answer the purpose well, and is easier to obtain clear after sterilising than the mash, or the worts from distilleries and yeast manufactories If special problems arise, as, for instance, the presence of certain species of micro-organisms, the analytical apparatus should be modified to suit the characteristics of the particular species Thus, for yeast factories, the appearance of moulds and putrefactive

bacteria will be of special importance Provision for the former can be made by cultivation of the water on the surface of congealed gelatine which has been mixed with a decoction of fruit, and for the latter, by employing ordinary plate-cultures consisting of neutral nutrient gelatine For special demonstration of the *Sarcina* forms in beer, a neutral decoction of yeast, with the addition of a small quantity of alcohol, will be found suitable. For developing "wild yeast," wort may be used with an admixture of hydrofluoric or tartaric acid.

Experiments may also be arranged by mixing the liquid with a certain quantity of the suspected water at different stages of the fermentation, the addition of pure yeast to the wort having been previously made The difficulty of this method when working with small quantities consists, as is well known, in approaching sufficiently near to practical conditions to make it possible to draw direct conclusions.

CHAPTER III

BACTERIA.

Bacteria occur in every shape, from the smallest specks or spheres to green algæ-like filaments, and they are found in nearly all possible localities, under the most varied conditions According to their action, a distinction is made between pathogenic, zymogenic, and chromogenic bacteria, or those that produce disease, fermentation, and coloration respectively

Our first knowledge of these living forms was obtained by placing small quantities of different substances under the microscope, and examining them with high powers In putrefying meat minute spherical bodies were found, which clearly multiplied by division, in sour milk short rods occurred, and in decomposing vegetable matter large spherical bodies and long fine threads, in the mucus of teeth very fine spiral threads were found Thus it was convenient provisionally to retain these forms, and to describe them as independent species Credit is due to Cohn for the first systematic classification of bacteria

Bacteria always consist of single cells. In their simplest form they occur as spherical bodies (*coccus*, Fig. 16, *a*) of varying size, often so small that they can only just be seen even with the strongest powers, and only give evidence of their existence as organisms during propagation by division If the coccus divides in one plane, *Diplo-* and *Streptococcus* are produced (*b*) (*c*) By division in two planes, the *Pediococcus* (*b*) is obtained, and by division in three directions the *Sarcina* form * If the cells assume a cylindrical form we have bacteria (*e*), which may be of very varied length. A distinction may be drawn between the spore-forming (*bacillus*) and the non-spore-forming (*bacterium*). When the rods are swollen in the middle, and thus form spindle shapes, we have the *Clostridium* form (*f*) If the cells are elongated, so as to become more or less thread-like, they are called *Leptothrix* (*g*), which may also occur as pseudo-filaments when several bacteria are grouped lengthwise, or as *Cladothrix*, when they lie close to one another and appear

* In the *Sarcina* forms that occur in beer, the division is incomplete in all three directions, but appears to vary, so that an irregular piling up of spherical bacteria takes place, or else a marked displacement of single cells is found Two spherical bacteria are often found strung together All such conglomerates of cells are surrounded by a gelatinous envelope, the development of which is dependent upon the nutritive conditions.

as irregular branching threads The filaments frequently assume wavy or spiral forms (*h*), when they are only slightly curved, we have the *Vibrio* form, when the spirals are more prominent, the *Spirillum* and *Spirochæte* forms, when they intertwine like a plait of hair, the form called *Spirulina* is produced

The thickness of most bacteria is about $\frac{1}{1000}$ mm (1 μ), the largest do not exceed 3 to 4 μ The thickness may, however, vary extraordinarily in one and the same kind, and the same is equally true of the length, because the cells elongate before they subdivide The limit may be placed at 10 to 12 μ, only filaments exceed this The conditions of nutriment play an important part in determining the size of any one species

In all probability bacteria exist of such small dimensions that they cannot be distinguished with existing microscopes Thus,

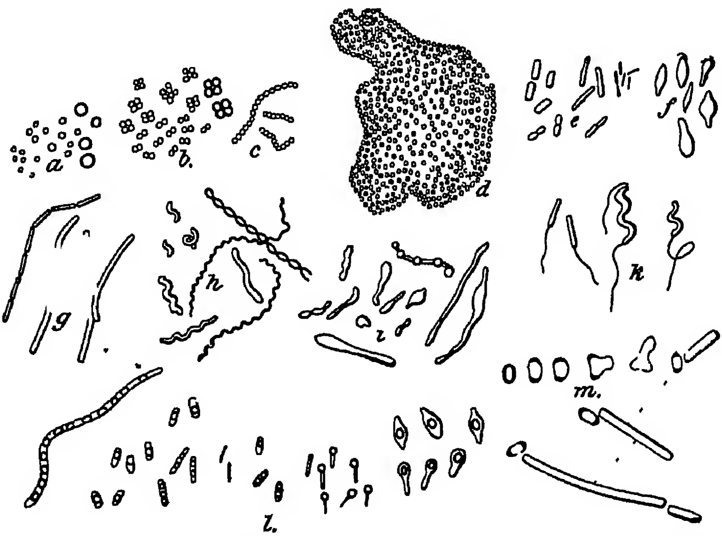


Fig 16 —Growth-forms of Bacteria (in part diagramatic)—*a*, Cocci, *b*, diplococci and pediococci (sarcina), *c*, streptococci, *d*, zoogloea, *e*, bacteria, *f*, clostridium, *g*, pseudo-filament, leptothrix, cladothrix, *h*, vibrio, spirillum, spirochæte and spirulina, *i*, involution forms, *k*, bacilli and spirilla, with cilia or flagella, *l*, bacilli, *m*, germination of the spore (*Bacillus subtilis*)

Roux has shown that in pleuro-pneumonia of cattle, an organism occurs which develops colonies on the substratum, while it is impossible, even with the strongest power, to see the individual bacteria

Lohnis investigated a large number of species (*e g*, *Bac subtilis*, *Streptococcus lactis*, *Bac. Bulgaricum*, *Sarcina flava*, *Azotobacter*), with results which, when compared with the observations of others, must be acknowledged to have a very wide bearing He established a developmental cycle of bacteria, which show a still more marked *polymorphism* than was formerly supposed. In all species examined

by him, the single cells were found, at a certain stage, to break up and merge into one another, thus forming what he calls a *symplasma*, a form which had been previously described and figured—*e g*, by Ray Lancaster (1876, in *Quarterly Journ Micr Science*, 16) The plasma is either amorphous or spherical and surrounded by a membrane (see later reference to mycobacteria and bacterial cysts), and in it are formed certain very small corpuscles, *regenerative units*, which on growing or amalgamating are seen to form vegetative cells of a new shape, or else they develop to more or less irregular *regenerative bodies*, in which normal cells are formed At this stage quite irregular ramifications sometimes develop, capable of multiplying through many generations, leading to marked polymorphous growths The regenerative bodies can multiply by division or budding, or they may merge into one another and form a symplasma Further, in ordinary bacterial cultures, cells of similar shape may enter into *conjunction*, two or a larger number uniting laterally or terminally, either directly or by bridge-like links. It has further been observed that bacteria not only multiply by division, but that more or less numerous *gonidia*, chiefly motile, appear in the cells (described by certain authors as "granular decay" of the cell). They may multiply in the cell, grow into new vegetative cells, or form buds or branches on the perforated wall of the mother-cell, they may develop as spores, or finally emerge as tiny independent cells These in turn may grow into larger spherical regenerative bodies before forming new cells, by division or expansion, or else the generative corpuscles may merge into large symplastic bodies

The gonidia-forming cells may also grow larger and form globular or spindle-shaped *gonidangia*, from which numerous gonidia emerge, or inside of which new vegetative cells may grow Finally, both vegetative cells and gonidangia are sometimes found to encyst like the symplasts The *regular stage of transition* is in every case the *symplastic* state, in all the phases of development, even that of spore-formation, the bacteria so unite or pass into the state described that the contents of the cells are mixed.

Thus, according to Lohnis' researches, each species shows forms and combinations, some of which were formerly described as particular species, or secretions in the medium having no connection with bacteria. Thus, even in ordinary technological research, it will be necessary to have more regard to polymorphism than has hitherto been the case

Isolated cells occur in the growth of many bacteria which differ from the rest in that they are irregularly swollen or branched, these have been named *involution forms* (Fig 16) In some species such cells represent a diseased growth—possibly a consequence of harmful ingredients in the culture medium—and they occur more particularly in old cultures, in other species, for instance the acetic

acid bacteria; they appear, on the contrary, to belong to a certain stage of the vegetative growth

The bacterial cells contain protoplasm, a homogeneous, feebly refractive substance, which may contain bright little granules. Occasionally one or more clear spaces are found within the cell, which by analogy with the higher plants are regarded as sap-cavities or vacuoles.

Thanks to improved methods, real insight has at last been obtained into the vexed problem of the presence of a true cell nucleus in bacteria We can only refer to the more recent work of A. Meyer, Vejdowski, Prazmowski, Rayman and Kruis, and Paravicini. Thus, in the young spores of *B. amylobacter*, A Meyer observed a roundish body, more refractive than the plasma and reacting chemically like the nucleus of fungus cells, while chemically distinguishable from plasma, fat, glycogen, and volutine, on which evidence Meyer considers it to be a nucleus. In *Bac. mycoides* and other species, after drying at room temperature, staining and bleaching, Rayman and Kruis observed distinct cell nuclei, which seem to participate in the division of cells, the nucleus being situated precisely at the place where the cell-wall has a stricture. Of particular interest are the observations made by Kruis on this bacterium, without staining, in broth culture, with ultra-violet light at 3,000 magnification, where the nucleus as well as the successive stages of division were distinctly seen On examination of the same preparation at the same magnification, but in daylight, the contents of the cells appeared to be entirely homogeneous *Azotobacter* was closely studied by Prazmowski, both young vegetative cells and spores, with vital staining and without staining. He describes the nucleus as a very refractive grain—the chromatic grain —lying in a slightly refractive basic substance, which is enveloped in a thin film. The successive divisions of the nucleus could be clearly observed by closely watching the development of a single cell. More recent observations of *B amylobacter*, *Streptococcus acidi lactici*, *Bac fluorescens non-liquefac*, and several other species, show that the nucleus plays the same part as it does in higher organisms; it proves, in fact, to participate in the formative functions of vegetative and reproductive life By examining cells that have not shrivelled, after fixing with a diluted mixture of acetic, chromic, and osmic acid, followed by coloration with hæmatoxylin—Paravicini observed the nucleus in *B. mycoides* and *megatherium* and, while the spore was forming, saw the plasma gather round the nucleus to form a thick membrane In the course of cell-division he first observed a division of the nucleus, whereupon the two daughter-nuclei were seen to take up positions at the two extremities of the cell, while the vacuoles merged into each other, and a partition-wall was formed in the middle These facts serve to prove that bacteria possess a true nucleus.

The granules that occur in the plasma are, without doubt, of varied character, even in one and the same cell. In many bacteria, fat globules have been distinguished with certainty, in sulphur bacteria, granules of sulphur occur as brightly refractive globules. Other granules occur in the plasma, which contain either starch or glycogen, especially before the formation of spores, they are used up as the spores develop. These granules are coloured either blue or reddish-brown on treatment with iodine.

Surrounding the protoplasmic body we find a **cell-wall or membrane.** By treatment with a hygroscopic substance, the inner surface of the cell-wall stands out clearly, owing to the contraction of the plasma. An examination by Loffler's method of staining generally shows that its outer layers have swollen up into a gelatinous mass, which becomes quite distinct when large numbers of bacteria are massed together. From a chemical standpoint it must be provisionally assumed that the cell-wall is differently constituted in different species In some it recalls the cellulose of the higher plants, whilst in others it appears rather to resemble the albuminoids in its properties.

In decomposing liquids, as well as in slime-fermentations, bacteria are frequently observed in which the outer layers of the cell-wall have been converted into a viscous condition, so that the mass of cells is embedded in a structureless slime Such structures are called *Zooglœa* When the slime has a sharply defined edge and doubtless a harder consistency, it has been described as a capsule formation, as, for instance, in the "frog-spawn" (*Leuconostoc*) of the sugar factory. Occasionally the outer layers of the cell-wall are transformed into a sheath of firm consistency, which eventually encloses a large number of independent cells (*Crenothrix*). The enclosed cells multiply inside the sheath until at last they force their way out, and the sheath survives for a time as an empty husk

At this point we may note the remarkable phenomena which Thaxter, Bauer, Quehl, and others have described. *Myxo-bacteria* —*i.e.*, swarms of organisms occurring particularly on excrement— which multiply by division, exhibit a slow crawling motion, and during their growth secrete a colourless slime, in which they live, and which enables the swarm to hold together The rods are eventually transformed into spherical spores, which form small red clusters, or else a number of small rods become enclosed in a common membrane, termed a cyst. The remarkable "*bacteria-bubbles*" (*bacteriocysts*) described by Müller-Thurgau occur in fruit wines containing tannic acid, especially in perry, and more particularly in and upon the yeast which settles after fermentation. These are zooglœa forms of lactic acid bacteria, which surround themselves with a membrane and thus resemble the cells of higher plants. Inside this membrane the bacteria are embedded in a

clear mobile liquid, and they eventually collect in the lower part of the bubble These may gradually increase in size. There is a certain resemblance between these and the remarkable forms described by Winogradsky in 1888, the *Amœbobacter* (sulphur bacteria), which also occur in cell families, the cells being bound together with threads of plasma, and the whole family moving as a slimy mass (amœbæ) in ever-varying forms. When these reach the resting stage, a thin gelatinous skin separates out, and is slowly transformed into a hard skin enclosing the whole family, which eventually breaks away from its husk.

Many bacteria contain blue, red, yellow, or green **colouring matter,** which may cause intense coloration In most bacteria of this category the colouring matter is present in solution in the nutritive liquid, whilst the bacteria appear to be colourless. In other cases, on the contrary, the colouring matter is found in the cells, for instance, in the red sulphur-bacteria, where the red colour plays the same part in the nutrition of the bacteria that chlorophyll plays in the higher plants One of the commonest pigment bacteria is the *Bacillus fluorescens liquefaciens* commonly occurring in water, which yields a greenish-yellow, fluorescent colour, soluble in water

The formation of colouring matter is in many bacteria dependent on temperature, supply of oxygen, and nature of nutrient medium. Thus, *B pyoceaneus* can produce the blue colour only in a peptone solution, and more particularly on addition of glycerine, whereas on a glucose medium no colour is produced In some bacteria an abundant development of pigment appears to be caused by treatment of an agar culture with a dilute solution of carbolic acid.

Shibata made the interesting observation that certain pigment bacteria which combine loosely with molecular oxygen—such as *B. brunneus*, *fuscus*, *Sarcina aurantiaca*—react in the same manner if they are killed or the colouring matter extracted with alcohol; combination with oxygen thus seems to be peculiar to the colouring matter. The colouring matters referred to belong to the so-called *lipochromes*, which are insoluble in water, but are soluble in alcohol and ether.

Phosphorescent bacteria are found more particularly in sea water; great numbers occur on dead animals and plants The phosphorescent phenomenon is connected with respiration, for it ceases when air is excluded

With regard to the **chemical composition** of bacteria, a number of analyses have been published Before the analyses were made, the growth was thoroughly washed to remove, as far as possible, every trace of the culture medium They show a content of about 85 per cent. of water, 8 to 14 per cent of albuminoids, 1 to 4 per cent. of fat and waxy bodies, and about 1 to 2 per cent of ash (sulphur, phosphorus, chlorine, potash, lime, magnesia, iron,

manganese, and silica) Their composition is, however, obviously influenced to a considerable extent by the nutriment. The slime formed by many bacteria is either a compound of a carbo-hydrate and an albuminoid, or a carbo-hydrate alone, as in the case of "frog-spawn" (*Leuconostoc*) and similar species Bacteria contain a number of enzymes of a more or less pronounced albuminoid character, and this is also the nature of the various poisonous substances which occur in several species

For **nutrition,** bacteria require carbonaceous and nitrogenous compounds, as well as the inorganic substances found in the ash. The majority of bacteria do not appear to possess the power of building up their organic constituents from inorganic material, they are dependent upon those organic compounds that have already been built up in animals and plants The nitrifying bacteria form an exception in that they can directly absorb carbon dioxide from the air, and the bacteria which occur on the nodules of the leguminosæ in like manner absorb nitrogen from the air and assimilate it.

It has been customary to distinguish between **Saprophytes** or organisms of putrescence and **parasites** which feed only upon living animals and plants. A corresponding classification of the bacteria has been attempted in a biological sense Alfred Fischer divides them into **prototrophic, metatrophic,** and **paratrophic** (from the Greek, *tropha*, nourishment).

By **prototrophic** bacteria are meant those, like the nitrifying, the iron and sulphur bacteria, which can take up these substances in an inorganic form The vast majority of bacteria are **metatrophic**; they utilise organic compounds of the most varied kind, while they promote putrescence or fermentation Lastly, the **paratrophic** are parasites; they do not occur in nature in a free state, but can only grow upon other forms of life. Nevertheless, it is possible to cultivate them—*e g*, in blood serum—at the temperature of the body.

The metatrophic bacteria, which form the vast majority, and are of special interest to the fermentation physiologist, are not equally responsive to the different carbonaceous and nitrogenous food-stuffs Peptone and amides are good sources of nitrogen. Many bacteria can also utilise ammonium salts and nitrates under given conditions, thus *Bact aceti* can assimilate ammonia in presence of acetic acid Similarly, according to Henneberg, certain species of acetic acid bacteria can utilise potassium nitrate and ammonium tartrate as sources of nitrogen, if the culture material contains sufficient dextrose

The carbohydrates constitute the most important source of carbon Of the different varieties of sugar, grape sugar forms an excellent food for bacteria

Adam found that a definite concentration of H ions in the

nutrient medium gives to each particular species the best chance of development, because it facilitates the metabolic process The "specific hydrogen number" is a constant He observed that in the *clostridium*-like bacteria but slight, if any, spore-formation takes place at the optimum, whereas, above and below the optimum, spores were seen to form freely. Adam observed that, under the simplest conditions, the addition of a definite proportion of acetic acid and sodium hydroxide is sufficient for the preparation of liquid nutritive media with different values of p_H The estimation of the specific hydrogen number is likely to be of some consequence in distinguishing between closely allied species

According to the conditions of nourishment, bacteria may bring about varying decomposition of the substrata

The different nutritive fluids and gelatines that are used in the culture of bacteria are described in Chap i., sec 6

Pasteur made the important discovery that there are certain bacteria and other micro-organisms which do not require free oxygen, but are capable of effecting active decomposition of the fermenting material, even when oxygen is excluded He, therefore, distinguished two classes of micro-organisms, *aerobic* and *anaerobic* Whilst the aerobic bacteria breathe in a similar manner to all other organisms, and thereby convert organic substances (non-nitrogenous) into carbon dioxide and water, and bring about similar decompositions with the nitrogeneous compounds, the anaerobic bacteria comprise on the contrary those whose life activity is sustained without free oxygen To this class belong some of the butyric bacteria, as well as the bacteria that ferment cellulose. Even small doses of free oxygen are apt to act as poison upon many anaerobic bacteria, more particularly on the vegetative cells, whereas the spores are only killed after prolonged action. The asporogenous species seem to be more resistant than the sporogenous forms

Since Pasteur's discovery (1861) numerous bacteria have been investigated in this respect, and it has been proved that there is every possible transitional stage between the obligatory aërobes (amongst which the hay bacillus, *Bac. subtilis*, must be classified) and the obligatory anaerobes A number of *facultative anaërobic* species are now known which grow well with access of air, but also develop, to a degree varying with the species, either in diluted air or even in the absence of oxygen, examples may be found among the lactic acid bacteria It is a well-established fact that with one and the same species the demand for oxygen varies according to the other life conditions. The heat-loving (thermophilous) bacteria form a typical example, for they can grow at a high temperature in the presence of air, whilst at a low temperature they grow only in the absence of air Of the obligatory aerobes, many, when well nourished, can develop in air containing only traces of oxygen.

Particular interest attaches to researches made by several authors (Chudiakow, Kursteiner, Ritter, and others), proving that *Clostridium butyricum* can support small doses of free oxygen without detriment to its development Chudiakow, by *adaptation* of this anaerobic species, made it thrive at pressures amounting to 50 mm But, on the other hand, this and other species, in experiments carried out with great care and accuracy, could be developed through numerous generations, without a trace of free oxygen By a similar adaptation it was possible to cultivate some facultative anaerobic species through a long succession of generations without a trace of free oxygen, and without prejudice to their specific functions, under such conditions, however, they seem to require definite sources of carbon, particularly dextrose

Whilst some bacteria are motionless (*e g*, lactic acid and certain acetic-acid bacteria), many species show a capacity for free movement, such bacteria are commonly met with in decomposing fluids This motion, which is not to be confounded with the Brownian molecular movement, usually consists of a forward swimming action, together with a rotation round the longer axis. The organs of motion, which only become visible on staining, consist of fine protoplasmic hairs—flagella or cilia—which are connected with the plasma of the cells through holes in the cell-wall. Certain species have only a single cilium, attached to one end of the cell, others have a bunch of cilia at one end, whilst cilia are distributed over the entire surface of others—*e g.*, the hay bacillus, in certain stages of development, and some of the common putrefying bacteria

By means of these organs of motion, bacteria are enabled to penetrate to that part of the nutritive fluid which offers the most favourable conditions for existence Thus, Engelmann has proved that aerobes move to the stratum of liquid which is richest in oxygen, whilst the anaerobes move in a contrary direction. Similarly, Pfeffer has shown that bacteria move to these parts of a fluid that contain nutriment of suitable concentration. The rate of motion is conditioned by temperature; thus, *Bac subtilis* moves more rapidly at 37° C. than at 20° C., whilst other bacteria cease to move at the former temperature

The **propagation** of bacteria takes place by **division.** It has been observed in detail in the larger species. The cells expand, fine transverse lines appear, which gradually increase in thickness and split into two leaflets, after this the organism separates into smaller rods, which sometimes remain united, sometimes become detached. Long before a trace of these transverse walls can be observed, staining will show that the organism consists of a series of segments, each of which corresponds to a subsequent individual. The newly formed segment cells are all in the same plane A

division in either one, two or three planes has been observed in certain cocci

In the case of many bacteria, formation of **spores** takes place in the following manner (Figs. 16, *l*, *m*, 24, *B*) The plasma in the cell becomes darker, and often distinctly granular, a small body subsequently appears—frequently at one end of the cell—strongly refractive to light, which quickly increases in size, and is surrounded by a membrane Meanwhile, by far the greater portion of the remaining plasma of the cell disappears, being used up in the formation of the spore This is seen enclosed in a clear liquid which gradually disappears, and finally the cell-wall shrivels up, and only remains as a withered appendage to the ripe spore. In many cases a swelling takes place in the mother-cell during spore-formation (Figs. 16, *l*, 24, *B*) Before spore-formation begins, the cells of many, especially anaerobic species, are coloured blue with iodine like starch-granulose Probably at this stage the cells store up reserve food material. The membrane of the spore is very strongly developed, and is frequently surrounded by a gelatinous envelope The contents are strongly refractive, and contain but little moisture. Spores are generally difficult to stain On the other hand, colouring matters once taken up by spores are retained better than by vegetative cells, and after bleaching, therefore, coloured spores become visible in a colourless cell. Usually only a single spore is formed in a cell.

One cause for spore-formation may be that during vegetative growth the products of its own activity—acids, alkalies, etc—accumulate in the nutritive substratum, and, as a consequence, further vegetative growth is checked. The exhaustion of the nutritive medium may produce the same effect Spore-formation demands a suitable temperature, and a certain amount of moisture. Spores are of value in enabling the species to survive when conditions occur that are unfavourable to vegetative life. They possess quite an extraordinary power of resistance to harmful influences. The membrane cannot be easily moistened or penetrated by water, and the great durability of spores is especially due to the fact that the plasma contains little or no moisture. Thus, according to Flügge, species occur amongst the peptonising bacteria of milk, the spores of which will withstand boiling for four hours. Spores of hay bacillus will also withstand boiling for hours. Spores can usually stand dry heat better than boiling in steam or water On the other hand, many spores show special resistance to heating in milk, and the same is true of neutral or feebly alkaline liquids, but less resistance is shown in presence of acid

As soon as favourable conditions of nutriment and temperature are established, spores germinate. They first swell up by absorption of water, and the contents lose their strong refractive power. A bacterium then grows out from the spore, the wall of the latter

is sometimes seen to burst or to unfold into two valves (Figs 16, 24). The full-grown rod then multiplies in the usual manner Spores may maintain their germinative power through a long period, sometimes for many years

In addition to the endosporic bacteria just discussed, "arthrosporic" bacteria were formerly described which do not form spores in the interior of the cell, but in which it was believed that members split off from vegetative cells form the starting point of fresh vegetative generations A microscopically discernible difference between the "arthrospores" and other cells, however, occurs only in a few cases, in that the walls of the latter thicken (Chlamydospores) Perhaps by continued investigation endogenous spores will be found in all such species

Temperature plays an important part in the life processes of bacteria We distinguish the minimum, optimum, and maximum temperature at which a particular vital function can be performed. These three cardinal points differ, not only for each species, but also for the individual functions of each, such as its rate of growth and its fermentative activity

Many bacteria are very resistant to low temperatures. J. Forster, B Fischer, Miquel, and others have shown that bacteria exist which multiply at the freezing point Certain species are not killed by exposure to a temperature of $-70°$ C, $-100°$ C, or even to the extreme temperatures of $-213°$ C and $-252°$ C. (Frisch, Pictet and Young, Macfadyen, and Rowland) In contrast to these, a number of **thermophilous** bacteria have been discovered. Miquel has described *Bacillus thermophilus*, which multiplies readily at 70° C, whilst its development is arrested at 42° C Other species will only develop at 60° C In the excrement of animals many species of frequent occurrence continue to grow at 75° C, whilst their growth is inhibited at about 39° C (*L. Rabinowitsch*) Certain lactic-acid bacteria and organisms occurring in molasses, in the fermentation of tobacco, and in the spontaneous heating of hay, belong to the thermophilous species The bacteria occurring in hay have been examined in detail by Miehe F. Cohn has also proved that the cause of the spontaneous heating of moist cotton waste is the presence of a micrococcus belonging to this group

With regard to the germicidal action of **light**, Downes and Blunt found, as early as 1877-1878, that direct sunshine powerfully restricts their growth, and that the most active rays of light are the strongly refractive blue and violet rays, well known to possess powerful photo-chemical properties. On the other hand, red and orange rays are less active, and heat rays which accompany the light rays possess no activity

H Buchner and S Bang, amongst more recent workers, have studied the action of light upon bacteria Buchner records that sunlight plays a part in the spontaneous purification of rivers by bacteria.

It is assumed that the effect of direct sunlight on bacteria is not entirely due to the action on the cells, but also to the alteration brought about in the substratum, whereby it becomes less suited for nutrition. For instance, the formation of hydrogen peroxide in nutritive agar by exposure to sunlight has been demonstrated A few quite exceptional bacteria are known which appear to thrive in bright light. This is true of the purple bacteria, which, like green plants, assimilate carbon in presence of light

Bacteria are capable of living at considerable depths Russel found bacteria alive at a depth of 1,100 metres They are, therefore, capable of withstanding a **pressure** of over 100 atmospheres, and certain putrefactive bacteria have survived a still higher pressure

With regard to the action of **antiseptics** on bacteria, the rule has already been laid down, in the section on sterilisation, that the higher the temperature the more easily they are killed. But, with regard to the restrictive action of antiseptics, it is usually weakest at the optimum temperature, and stronger at both higher and lower temperatures Very dilute solutions of an antiseptic may encourage the growth of bacteria

Various species react differently to the same concentration of a reagent, and the action depends to a great extent, with any given species, upon the state of nourishment of the cells Spores are much more resistant than vegetative cells It has proved possible to propagate bacteria in the presence of successively increased quantities of an antiseptic, but the characters so obtained prove not to be fixed, but disappear as soon as the culture is prepared in a substratum free from poison

Highly interesting results, which should have a wide application, were arrived at by Richet in his extensive researches on lactic acid bacteria By suitable treatment they can adapt themselves to a large number of poisons Thus, by addition of increasing doses of thallium nitrate, cultures could be made to grow in milk containing 1·5 g per litre, but not all at once During the first eight days the bacterium grows weakly, but on the ninth day it suddenly begins to adapt itself, and on the twelfth day the strange phenomenon is observed, that the ferment has acquired the power of growing more freely and accordingly producing a larger amount of acid in presence of the poison than it did in normal milk Thus the adaptation requires a certain length of time, but it nearly always takes place by *sudden mutations* Thus, if an untreated bacterial culture is sown in a large number of flasks containing potassium arsenate, as an addition to the nutritive solution, the growths will develop very imperfectly, yet one or two of them will usually be found to grow very freely, so as to produce a degree of acidity 2 to 3 times as high as that observed in the other flasks, in other words, the vegetation in the toxic solutions shows much

greater variation than the vegetation in the normal solutions, the ferment, under unknown influences, has suddenly acclimatised itself in some of the flasks, and has undergone in these a *sudden mutation*

Bacteria and other micro-organisms when subjected to **mechanical** vibration behave very variously Horvath proved that gentle vibration has no action on their growth, whereas violent shaking hinders or entirely inhibits it Meltzer arrived at the conclusion, after prolonged experiments with liquid cultures, that gentle vibration promotes the multiplication of micro-organisms, with a given degree of motion the rate of germination of the species is at the maximum, whilst any stronger vibration restricts it The optimum and maximum differ for each species According to Appel, cultures of bacteria on solid substrata behave the same whether they are shaken or not

Attempts have been made ever since the discovery of bacteria to define this large group of organisms, and to classify the various species in one system, like other sections of the vegetable kingdom.

With the exception of a small number of doubtful forms, amongst which *Crenothrix* may be named, the bacteria form a fairly uniform group, exhibiting the same simple structure and the same method of propagation throughout It is an interesting fact that amongst the lowest green plants, the algæ, there is a group which exhibits the same construction and the same method of propagation as the bacteria, so that the lowest green plants connect up with the lowest fungi It has already been mentioned that green plants possess the power of absorbing and assimilating the carbon dioxide of the air, owing to a special constituent of the cells, chlorophyll. This power is not possessed by fungi, and the lowest group of algæ has been classified, therefore, under the name of *Schizo-algæ*, in contrast to the *Schizo-fungi* It is not only a physiological difference that distinguishes the two groups—that could not be used as the basis for a systematic classification—but rather that the structure of the cell contents is entirely different, since the *Schizo-algæ* contain grains of chlorophyll On the other hand, the *Schizo-algæ* have not the power possessed by bacteria of forming endogenous spores, but the method of division exhibited by *Crenothrix* (see Fig 31) frequently occurs in the algæ To illustrate how difficult it is to define strict limits in nature, it may be stated that there are undoubted bacteria which contain green colouring matter, and yet others that can assimilate atmospheric carbon dioxide.

It is generally agreed that bacteria are to be classed amongst the fungi, although, in their method of propagation, they stand nearer to the algæ. There are, however, fungi which show the same kind of cell division as bacteria, and even the remarkable formation of endospores also occurs amongst many fungi,

especially amongst those that will occupy us next, the *Saccharomycetes*.

We have seen that bacteria have points of contact, both with the lowest forms of algæ and also with the lowest forms of fungi It must also be noted that in so far as certain bacteria move about with the help of cilia, a similar relationship exists between them and the lowest group of the animal kingdom (the flagellata) This forms a third point of resemblance

Cohn, in 1875, first published a system of bacteria, this must now be regarded as out-of-date, since Zopf, de Bary, van Tieghem, Hueppe, and many others have established new divisions, which correspond more closely to the natural boundaries

Any botanical classification of the bacteria will necessarily offer the same advantages and the same drawbacks as the classifications attempted for the higher plants The deficiencies stand out in bolder relief in our field, inasmuch as bacteria do not usually exhibit conspicuous morphological differences, and also because one and the same species may assume various shapes in successive stages of development, so that transition forms are frequently observed between the genera and even between the larger groups. Nevertheless, it has been found possible to survey the numerous species more readily by establishing a provisional botanical classification; a large number of bacterial species can naturally be grouped in large characteristic families. The best authorities disagree how to allot the genera to each family.

The main lines of Lehmann and Neumann's scheme may be taken as an example of a botanical classification.

I Coccaceæ (Zopf). Migula. Spherical Bacteria.

Cells in the free state mostly spherical, division in one, two, or three planes Endospores very rare. Flagella rare. Before division the length of the cells may be $1\frac{1}{2}$ times the breadth

(1) **Streptococcus** (Billroth) —Cells divide only at right angles to the direction of growth. When they remain connected, they form chains, often with the cells linked in pairs Instead of chains, sometimes occur as pairs of cocci

(2) **Sarcina** (Goodsir).—Cells divide regularly in three planes, at all events on suitable nutrient media, and remain attached in larger or smaller cubic packets.

(3) **Micrococcus** (Cohn) —Cells divide irregularly in different planes, forming bigger or smaller packets, cells sometimes grouped in pairs, threes and fours To this family belong the genera Staphylococcus, Pediococcus, Merismopedia

The definition of these three genera is rather artificial, many transitional forms have been observed.

II. Bacteriaceæ (Zopf). Migula. Rod Bacteria

Length of cells at least 1½, but mostly 2 to 6 times the breadth, straight or curved in one plane only, they sometimes form long threads or apparent threads Division across the longitudinal axis after expansion of the rod

(1) **Bacterium.**—Without endogenous spores Rods generally less than 0 8 to 1 μ thick

(2) **Bacillus.**—With endogenous spores Rods often more than 1 μ thick

The spore-forming rods are closely related to one another.

III. Spirillaceæ Migula Spiral Bacteria

Vegetative bodies single-celled, arched or spirally twisted; more or less expanded, division at right angles to longitudinal axis. Cells often form short chains consisting of few links, very often in pairs, mostly in lively motion by action of terminal flagella. Formation of endospores very rare.

(1) **Vibrio.**—Cells short, slightly arched, rigid, curved in form of a comma, sometimes hanging together in cork-screw masses, generally with one, rarely two, terminal flagella

(2) **Spirillum.**—Cells long, spirally curved, cork-screw shaped, rigid, bushy flagella, mostly polar.

APPENDIX I.—Actinomyces. Lachner-Sandoval.

(Hyphomycetes allied to the Schizomycetes)

Organisms destitute of chlorophyll, consisting of thin threads with true branching, sometimes even abundantly branched mycelium, partially with formation of conidia. Young cultures frequently show only Schizomyces forms, non-branching rods, in no way distinguishable from ordinary Schizomycetes (mucous bacillus, diphtheria bacillus, tubercle bac , Actinomyces)

APPENDIX II —Higher Schizomycetes

Are closely allied to the chlorophyllous algæ No true branching In many species there are formed, in some few cells, zoospores provided with flagella

(Filaments without distinct sheaths —Leptothrix, Beggiatoa, with granules of sulphur. Filaments with sheaths —Chlamydothrix, Crenothrix, Cladothrix, Thiothrix, with granules of sulphur)

Rahn tried to group the different species according to their

resemblance, without adopting any definite principle of division, but with particular regard to **transitional** forms and to **physiological** properties The species are grouped in three great classes, where those of most frequent occurrence are set up as chief species, namely —

(1) **Sporing Rods** (*Bacilli*), divided into sub-groups, aerobic and anaerobic

(2) **Non-Sporing Rods** (*Bacteria* and *Streptococci*).

(3) **Micrococcus.**

For details, reference must be made to Rahn's interesting and very explicit memoir Orla Jensen adopts as a classification of the non-motile bacteria their behaviour, not only towards the sugars, but also towards nitrogenous compounds, the fact being that the selection made by a bacterial species among the nitrogenous foodstuffs often determines that made among the sugars. In regard to the motile species, he uses as a basis of classification the disposition of the cilia, which seems to bear a certain relation to the physiological properties of the bacteria, inasmuch as those species which can live upon inorganic nutriment alone, deriving their vital energy from simple oxidation processes, have terminal cilia, while those which require comparatively complex organic foodstuffs have their cilia distributed all over the cell.

The definition of each species is surrounded by considerable difficulties, owing to the extraordinary **variability** to which all bacteria are subject when the external conditions of life change Thus, coloured races transferred to other media turn white, acid forming turn into such as form no acid, anaerobic into aerobic bacteria, and so on. The consequence is that one and the same species has often been characterised differently and named afresh. The excessive splitting up into new species has rightly been abandoned in the light of modern observations, and we now describe as one species many forms previously considered to be independent species, where by suitable cultivation the supposed distinctive characters have disappeared. Further, there is a *tendency* to group the revised species in **types,** having certain arbitrarily chosen properties and characters in common Billroth, Nágeli, and others held that no well-defined species exists, since so many morphological as well as physiological transition forms are met with. At any rate, the immense number of observations made since their time goes to prove the existence of more or less close **affinities** between many of the species described

In the present work it has been necessary to limit the system in a rather arbitrary manner, as we have only to deal with those bacterial species with which the fermentation industry is concerned.

1. Acetic Acid Bacteria.

The acetic fermentation is a process of oxidation. By the activity of the bacteria in presence of oxygen, the alcohol in the liquor is oxidised, as a rule, according to Neuberg and Nord, to acetaldehyde and water, and then the aldehyde is further oxidised to form acetic acid. It is a process differing greatly from those classed as fermentations, and has a certain similarity with the process of breathing The entire content of alcohol undergoes change without the production of by-products, the bacteria can, however, bring about the combustion of acetic acid to form carbon dioxide and water It is, therefore, of importance to interrupt the process as soon as acetic acid is formed, if the full yield is to be obtained

Persoon, as early as 1822, was acquainted with the vegetable character of the film which forms on the surface of liquids undergoing acetic fermentation, and he named the film *Mycoderma*

In 1837-38 the view was also expressed by Turpin and Kutzing that the acetic acid fermentation is caused by a micro-organism, which Kutzing described and delineated under the name of *Ulvina aceti* Starting from this, Pasteur, first in his treatise of 1864 and subsequently in his work, *Études sur le vinaigre*, in 1868, furnished experimental proof of the correctness of this view He sowed a trace of the film on a mixture of wine and wine-vinegar, and thus obtained a stronger development of acetic acid than was possible by allowing the liquid to undergo spontaneous fermentation, and on this he based a process for manufacturing vinegar. He assumed that the acetic fermentation was caused by a single species of micro-organism, which he called *Mycoderma aceti* As early as 1879, E C Hansen discovered that at least two distinct species are concealed under the name of *Mycoderma aceti*, which now go by the names of *Bacterium aceti* and *Bact Pasteurianum;* and now a whole series of species are distinguished To obtain the best results in this branch of industry, it is necessary to start with an absolutely pure culture of a methodically selected species

Whilst Pasteur does not explicitly maintain in his memoir that the oxidation of alcohol to acetic acid brought about by bacteria is a purely physiological process, von Knierim and Adolf Mayer expressed this opinion, the correctness of which the latter confirmed by proving that the vinegar film exercises its greatest activity at 35° C, and that it ceases to react at 40° C, and further that the film cannot react on more than 14 per cent alcohol (Landwirtsch Versuchsstat, 16, 1873) The purely chemical action of platinum black on alcohol presents a contrast, for it is able to react at higher temperatures and with high concentrations A. J. Brown observed that *B. aceti*, in contradistinction to platinum-black, does not attack either methyl alcohol or erythritol

Pasteur showed that the acetic acid generated by the oxidation of alcohol is transformed, if the oxidation is continued, into carbon dioxide and water This has been confirmed by A J Brown

A. J Brown, Boutroux, Bertrand, and Watermann found that acetic acid bacteria are able to form *gluconic acid* from glucose and allied sugars, but not from lævulose The different species, however, behave differently in this respect, as also in their power of inverting cane-sugar (*e.g*, in presence of yeast-water) From this sugar they do not form acid (although they do from a mixture of glucose and lævulose), and it, therefore, seems to be a legitimate conclusion that cane-sugar can be assimilated direct (Watermann)

An important advance was made in our knowledge of acetic bacteria when Buchner and Meisenheimer, as well as Herzog, proved that this remarkable fermentation is brought about by the activity of an enzyme. The cells may be killed with acetone, and then treated in the same way as the alcohol yeasts (see Chap. v), and it can then be shown that, after evaporating the liquid, the residue can bring about the acetic fermentation, although it contains no living cells By this discovery the real nature of the fermentation becomes clear Like the alcoholic fermentation, it is caused by an enzyme, which may react independently of the living cell that brought it into existence

These bacteria grow vigorously in many nutritive fluids—*e g*, in dextrose solution with peptone and salts The presence of alcohol is not an essential condition of their existence, and, indeed, more than 4 per cent of alcohol acts restrictively on their growth.

According to Janke, the following nutrient solution is well adapted for the growth of acetic bacteria —Per litre—0 4 g. K_2HPO_4, 1 g. $(NH_4)_2HPO_4$, 0 4 g $MgSO_4 + 7H_2O$, 5 c c glycerine, 1 g succinic acid (to dissolve the precipitate), and 3 per cent alcohol. The ammonia salts are specially valuable nutrients For micro-organisms occurring in the "quick vinegar process" a mixture of thin vinegar with diluted beer-wort, beer, or grain-mash is satisfactory.

To produce a spontaneous growth of acetic acid bacteria, beer may be allowed to stand with a mixture of cane-sugar and acetic acid (50 c c + 2 g + about 3 c.c) in open glasses at 30° to 35° C At this temperature *Mycoderma*, which is otherwise sure to make its appearance, is usually suppressed.

For cultivating and characterising the different species, Beijerinck (*Cent f Bakt*, 2 Abt., 4, 1898) used 100 c c potable water + 3 c c. alcohol + 0·05 g amm phosphate + 0 01 g. potass chlorate This is a liquid in which many species are not able to form films—*e.g*, *B. aceti* does form a film, *B Pasteurianum*, *B. rancens*, *B. xylinum* do not.

B. aceti was shown by Bertrand and Sazerac to effect the conversion of alcohol into acetic acid far more quickly if the

nutrient liquid contains a trace of *manganese* An addition of 1 10,000 of manganese sulphate to a nutrient solution of yeast decoction, albumen and alcohol, containing 5 g. of dry extract per litre, brought about most quickly the oxidation of the alcohol. If larger or smaller quantities were used, the action was retarded or completely arrested A similar effect is brought about by uranium acetate in a dilution of 1 500 (Agulhon et Sazerac, *Compt rend., Ac. d sc*, 155, 1912).

Mezzadroli finds that acetic acid bacteria, if kept in common nutrient gelatine and gelose, lose their power of forming acetic acid, and recover it but slowly when grown in a liquid of suitable composition The most active species in the manufacture of acetic acid were also found to attack nitrogenous compounds with the greatest vigour. He suggests that the cultures should be preserved in a liquid such as that recommended by Pasteur, consisting of yeast-water, 2 per cent alcohol, and 1 per cent. pure acetic acid. In such a solution they will keep their enzyme intact, but, to keep them alive, frequent inoculation is necessary.

Söhngen found that bacteria occurring in the "quick vinegar process" and grown with limited access of air (*e g*, in Erlenmeyer flasks) are rapidly enabled to produce an increased amount of acid, if colloids (blood charcoal, peat, blotting paper, silicic acid) are added to the nutrient medium

Hansen's researches are among the first which proved that a definite fermentation is not induced by one species of bacterium only, but by several, these researches also furnish some of the earliest experimental evidence of the fact that one and the same species can occur in very different guise, the correctness of his results was later confirmed by Zopf, de Bary, and A J. Brown By means of his staining experiments with *Bacterium* (*Mycoderma*) *aceti* (1879), he discovered that at least two distinct species are hidden under this name, of which the one, like most other bacteria, is stained yellow by iodine, whilst the other assumes a blue coloration with the same reagent. For the former he retained the old name *Bact aceti*, whilst the one stained blue he named after Pasteur—*Bact. Pasteurianum.* The film formations on wort and beer, likewise growths on wort-gelatine, give a fine blue colour with tincture of iodine, or iodine dissolved in a solution of potassium iodide, whilst growths which develop on yeast-water and on broth with peptone and gelatine are coloured yellow; even very old films on beer show a yellow reaction It is the slime formation secreted from the cell-wall that is coloured blue At a later period, Hansen discovered a third species.

These three species are characterised as follows.—*Bacterium aceti* (Hansen) (Fig 17) forms a slimy, smooth film on "double beer" (top-fermentation beer, rich in extract, containing 1 per cent. of alcohol), at a temperature of 34° C. and in the course of

24 hours. The slime is not coloured by iodine. The cells of this film consist of rod-bacteria, hour-glass-shaped, and arranged in chains, occasionally longer rods and threads occur, with or without swellings. At 40°-40·5° C. long thin threads develop. In plate-cultures with wort-gelatine at 25° C. these bacteria form colonies with sharply defined edges, or, more rarely, stellate colonies, which appear grey by reflected light, bluish by transmitted light, they mainly consist of single rod-bacteria. In peptone-gelatine broth the colonies are surrounded by milky zones, separated from them by clear zones, they may later become iridescent. On sowing drops on wort-gelatine, flat, spreading, rosette-shaped colonies are formed at 25° C. in the course of 18 days. In "double beer" the maximum temperature for growth is 42° C., the minimum 4°-5° C.

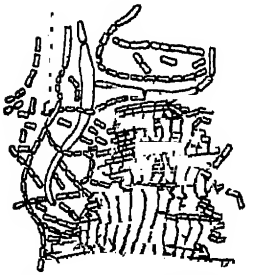


Fig 17.—*Bacterium aceti* (after Hansen)

This species is of common occurrence both in high- and low-fermentation beers.

Bacterium Pasteurianum Hansen (Fig. 18) forms a dry film on "double beer" at 34° C., which soon becomes wrinkled and pleated. In young, vigorous films on beer or wort, at favourable temperatures, the slime surrounding the cells is coloured blue by iodine. The cells of the film form long chains, and are, on the average, larger, especially thicker, than in the previous species. The thread-like form at 40°-40·5° C. is also a little thicker than that

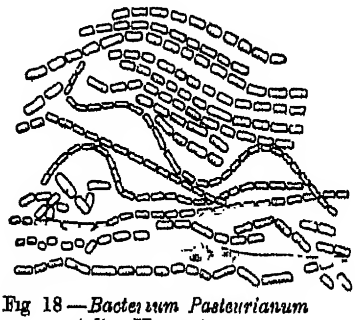


Fig 18.—*Bacterium Pasteurianum* (after Hansen)

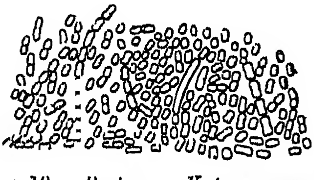


Fig 19.—*Bacterium Kutzingianum* (after Hansen)

of *Bact aceti*. In plate-cultures, with wort-gelatine at 25° C, the colonies resemble those of the previous species, but are a little smaller, and consist chiefly of chains. In peptone-gelatine broth the colonies are similar to the previous species. On sowing drops on wort-gelatine wrinkled colonies develop at 25° C. in the course of 18 days, which are slightly raised, and present a sharp outline or one slightly jagged. In "double beer" the maximum temperature for growth is 42° C, minimum 5°-6° C.

This species is more frequently met with in high- than in low-fermentation breweries

Bacterium Kützingianum Hansen (Fig. 19) forms a dry film, on "double beer" at 34° C, which creeps up the side of the flask The slime is coloured blue under the same conditions as *Bact. Pasteurianum*. The film consists of small rod-bacteria, which are most frequently single or connected in pairs, and seldom form chains The thread form at 40°-40·5° C presents almost the same appear-

Fig 20 —*Bacterium Pasteurianum*—The thread form after cultivation for 24 hours on "double beer" at 40°-40 5° C (after Hansen)

ance as that of *Bact Pasteurianum*. In plate-cultures with wort-gelatine at 25° C the colonies are analogous to those of the previous species They consist almost exclusively of small, single rod-bacteria. In peptone-gelatine broth the colonies resemble those of the two previous species. On sowing drops on wort-gelatine at 25° C., colonies develop in the course of 18 days resembling those of *Bact. Pasteurianum*, but with a smooth surface without wrinkles.

On "double beer" gelatine these colonies are slimy, whilst in the two previous species they have a dry surface. In "double beer" the maximum temperature for growth is 42° C, minimum 6°-7° C.

This species was discovered in "double beer"

Hansen's thorough investigation of acetic acid bacteria has assumed great importance in the general biology and morphology of bacteria, owing to the light thrown on one of the factors causing multiplicity of bacterial forms

Fig 21.—*Bacterium Pasteurianum*.—Transformation of the thread-forms into swollen forms and chains after cultivation in "double beer" at about 34° C. (after Hansen)

Each species of the acetic bacteria examined by Hansen occurs in three essentially different forms dependent on temperature—chains, consisting of short rods, long threads, and swollen forms. If sown on "double beer," which is very favourable to their growth, the various species give a growth consisting of chains at all temperatures from 5°-34° C, which develops well, notably at 34° C. If a bit of this young film is transferred to fresh nutritive liquid at 40°-40·5° C., the cells grow into long threads in a few hours (Fig. 20).

In some species, these threads can attain a length of 500 μ * and more, whilst the links of the chain measure only 2 to 3 μ. If the culture of long threads is then placed at a temperature of 34° C., a transformation into the chain form again takes place. Whilst developing at this temperature, the long threads increase, not only in length, but also in thickness, and that often very considerably. Thus an endless variety of polymorphous swollen forms are produced (Fig 21). It is not till then that the threads divide into small links, giving rise to typical chains Only the thickest parts of the swollen threads remain undivided, and are at last dissolved Thus the swollen forms play a regular part in the cycle of changes. This cycle furnishes a striking example of the effect of temperature in determining the form assumed by bacteria

The species *Bacterium aceti* and *Bacterium Pasteurianum* differ, according to Lafar, both chemically and physiologically. In sterilised beer they give different fermentation reactions At higher temperatures, *Bact Pasteurianum* acquires a higher acidifying power than *Bact aceti*, on the other hand, *Bact aceti* is able to carry on a vigorous fermentation at 4°-4·5° C, whilst at this temperature *Bact Pasteurianum* forms no appreciable amount of acetic acid At 33°-34° C *Bact Pasteurianum* reaches the maximum of acetic acid formation (3 3 per cent by weight) in seven days, after which the formation slowly diminishes, and finally ceases. After the maximum acid formation has been reached, irregularly swollen cells make their appearance in the culture, which, under the existing nutritive conditions, may probably be considered as diseased or degenerate forms (involution forms)

Bacterium xylinum is essentially different from these three species It was described by Adrian J Brown in 1886, who examined it especially from a chemical point of view It forms a film, the slime of which becomes cartilaginous and tough like leather The growth, consisting of motionless rods resembling *Bact aceti*, gradually fills up the whole liquid This species is essentially different from the three first described, in another respect —the slimy envelope shows the cellulose reaction, which is not the case with the slime of Hansen's three species. According to Emmerling the slimy sheath contains an albuminoid substance resembling chitin

It was demonstrated in the author's laboratory that this species occurs in vinegar factories, in many countries, in a vigorous state of development According to Henneberg, it may react unfavourably on the aroma of vinegar, and by forming slime, may arrest the quick vinegar process

The sorbose bacterium investigated by Bertrand, which causes the conversion of sorbite from the juice of mountain ash berries (*Sorbus aucuparia*), into sorbose, is identical with *Bact xylinum*.

* 1 μ = 0 001 millimetre

A species of very frequent occurrence in English beers, *B. aceti viscosum*, which provokes a marked ropiness, was studied and described by Baker, Day, and Hulton. It forms motile or non-motile, short rods, generally 1·2 μ long and 0 5 μ thick and usually united in pairs, in some cases, single elements or chains are found Involution forms rare No spore-formation Optimum temperature for growth 20°-25° C Grows vigorously in a glucose-peptone solution To unhopped wort, it first imparts a uniform turbidity; a ring is then formed, grey and gelatinous and a slight grey submerged gelatinous film, gradually a ropy sediment forms, and the liquid becomes fairly clear but decidedly ropy. The latter property disappears in a few weeks On wort-agar an almost transparent, grey, wet streak is produced, raised, with smooth edge, neither gelatinous nor ropy, but suggesting both On wort gelatine the surface colony is of a somewhat purple colour, with a radiating structure Regarding the conditions in which the disease is apt to be provoked, the authors state that the bacterium is more prone to cause ropiness in beer, if the infection takes place prior to fermentation A higher acidity and typical ropiness are brought about if air has access to the beer during the development of the bacteria With high acidities, the ropiness disappears All beers are not equally susceptible to the infection Varying the quantity of hops, or the amount of carbohydrates and soluble protein, within reasonable limits, is without effect on the growth of the bacterium It has not been possible to ascertain the conditions causing the production of ropiness of this kind In a few samples of ropy English beer, the authors discovered another species—short rods—which produces ropiness from *nitrogenous* compounds, independently of the carbohydrates and polyhydric alcohols It is incapable of infecting beer directly, but, if wort be infected, it may survive fermentation, nevertheless a deleterious effect could only be observed if the wort was strongly infected

A group of **slime-forming** acetic acid bacteria—the **Hanseanum** group—were observed by Janke in Vienna beer They form on beer and yeast water ropy or glutinous films, in which they chiefly occur as short rods, single or in pairs, motile, and more rarely in other forms On solid media they form a greyish-white, translucent, moist and raised coating One of the types is coloured slightly yellow by iodine, and acidifies glucose, the other assumes a brownish-violet colour and does not acidify glucose, but saccharose These bacteria, like those occurring in the "quick vinegar process," only require ammonia salts for their nitrogenous diet, contenting themselves with acetic acid as their source of carbon. They are able to resist higher acidities than most of the species existing in the liquor A particular group of acetic acid bacteria, which are also apt to cause ropiness, are mentioned in the chapter relating to slime-forming bacteria.

In a film of beer left to stand at 20°-30° C., Beijerinck found a very peculiar species—*Acetobacter melanogenum*—which forms brown colonies on wort-gelatine and makes the gelatine insoluble in boiling water, or when acted upon by trypsin. Iron salts are coloured bluish-black In beer it produces an abundant formation of acetic acid

Zeidler found an acetic acid organism in lager beer, *Termobacterium aceti* or *B Zeidleri*, which occurs as short motile cells and involution forms When a given quantity of acid has been formed in the liquid, movement of the cells ceases If the culture is sown on hopped wort, a cloudy turbidity forms on the surface; the whole liquid gradually becomes turbid, and acquires a yellowish-brown colour. Small films form on the surface of the liquid, which soon sink, and thus a loose brownish deposit is produced The species does not appear to be dangerous in the brewery In beer, the culture is destroyed at 35°-40° C

Bacterium oxydans, a species with motile cells, was described by Henneberg He discovered it on low-fermentation beer which had been standing in vessels at a temperature of 25°-27° C It forms roundish colonies on gelatine, which later assume irregular shapes with ramifications On sterilised beer it forms a delicate film, consisting of separate prominences creeping up the sides of the vessel In its younger stages the film consists of pairs of cells, later, of long chains. Beer is rendered turbid by this species. In its younger stages, motile cells have been observed. At a temperature of 36° C. the growth on beer consists almost exclusively of long, uniform threads This species also shows the irregular, swollen forms, as, for instance, on beer at 26° C. The cells are not coloured blue by iodine The optimum temperature for growth lies between 18° and 21° C The upper limit of temperature for the formation of motile cells was found to be 37°-40° C (or 44° when rapidly heated). The temperature at which the culture is destroyed lies between 55° and 60° C. for moist heat, and between 97° and 100° C for dry heat. The oxidation of alcohol into acetic acid has its optimum between 23° and 27° C

This species oxidises a great variety of carbon compounds

Henneberg subsequently described the following species or varieties —*Bact acetigenum*, which occurs in the quick vinegar vats, forms small, rounded, swarming cells, which are not linked in chains At a later stage swollen cells may appear The species forms a thin, matted and very tough film, which finally sinks to the bottom in isolated patches, giving room for a new film formation By treatment with iodine and sulphuric acid, a blue coloration may take place The acetic acid produced by this species is very aromatic, owing to the formation of ethyl acetate. The species has its optimum at 33° C

Bact acetosum, which is found in high-fermentation beer, forms

long chains, and, at the same time, irregular shapes. The film is solid, dry and after a time, wrinkled, the liquid is clear. The optimum for this species is about 28° C

Bact industrium occurs as short swarming cells without chain formation or irregular shapes On gelatine it forms greyish-white slimy colonies, in liquids, a thick slimy film and a solid ring attached to the side of the vessel When shaken, the film flocculates The liquor is rendered turbid The optimum on wort-agar is 23° C. The upper limit of temperature for motile forms is about 45° C. The species oxidises a large number of compounds The vinegar produced contains much aldehyde *

Bact ascendens, which is found in wine and wine-vinegar, likewise consists principally of single cells or pairs of cells, but also forms chains On grape-sugar-gelatine and grape-sugar-agar the colonies are surrounded by a white raised ring In liquids the species forms a very delicate uniform film, which creeps to an extraordinary height up the sides of the vessel The film is easily broken up and forms a flocculent deposit, and the liquid is rendered turbid On wort-agar the optimum is 31° C This species is only capable of oxidising a minute amount of material The vinegar produced is distinguished by its odour of acetic ether In old cultures the vinegar has a very pungent odour

Amongst sub-species, or varieties, which occur in the quick vinegar process, Henneberg has isolated the following —

Bact Schützenbachi, which occurs as round, oval, or longish cells, often also as sickle-shaped or irregularly bent and inflated forms, sometimes single and sometimes in chains On wort gelatine it forms round, clear, glistening colonies with a yellowish-brown centre On beer-gelatine the old colonies have a whitish granular surface. The very thin film that forms on liquids easily sinks to the bottom as granules The optimum appears to lie between 25° and 27·5° C

Bact curvum has rounded, longish, oval or elongated cells, with either rounded or pointed ends The more or less curved cells are especially characteristic, it also forms chains On wort-gelatine the colonies are round and transparent, with a raised edge and projecting centre, and frequently have a whitish dry appearance The film forming on liquids easily sinks to the bottom. The optimum lies between 25° and 30° C

Bact orleanense.—The cells vary in shape from spheres to pronounced rod-like forms, with all possible transitions The rods are straight or bent, single or linked in chains, swollen cells also occur On wort-gelatine irregular whitish colonies develop On beer-gelatine the older colonies are reddish in colour, with a moist glistening surface The film on liquids adheres firmly; the liquid,

* Beijerinck and Hoyer group *B acetosum, B oxydans, B industrium,* and *Termobact. aceti* in *one* species, *B rancens.*

therefore, remains clear The optimum is at 30° C, and, later, between 20° and 30° C The species may be used either for the quick vinegar or the wine-vinegar process

The same author has described the following special wine-vinegar bacteria (Orleans process) —

Bact xylinoides occurs both in the form of spheres and of short or long rods, they may be straight, bent, or irregular and sometimes swollen, single, or in chains On wort-gelatine the colonies appear like drops of water and often display a light brownish nucleus On beer-gelatine they have a moist, glistening, pale-brownish surface The film formation on liquids fluctuates greatly On sugar-yeast-water and on beer it is thick and tough, like *Bact. xylinum*, on other liquids it may exhibit every transition from a thin, dry, or smooth, to a thick, rough covering. The thick films give the cellulose reaction on treatment with iodine and sulphuric acid, but the thin do not The optimum for agar-cultures is at 28° C, and later at 20°-23° C In wine-vinegar mash the optimum lies nearer 24° than 28° The species is found widely distributed throughout wine-vinegar factories It can be distinguished from *Bact xylinum* by the multifarious kinds of skin growth

Bact vini acetati has rounded, oval and but seldom moderately elongated cells, single or linked in pairs and triads Inflated cells also occur The colonies on wort-gelatine are round, clear, with a glistening moist surface and a whitish deposit in the middle The films are not very coherent and the culture liquid soon turns cloudy On wort with 3 per cent alcohol, the film has a greasy appearance and pale yellow colour The optimum is at 28° to 33°

Finally, an acetic acid bacterium may be mentioned, found by J C Holm on the cocoa bean, which he named *Bact. aceticum rosaceum* It forms short, rounded, motionless rods, 1·6 μ in length, single or in pairs On wort or beer it forms a very weak, pale-coloured film, whilst the colonies on wort-gelatine and agar are distinctly pink

The production of acetic acid on a large **industrial scale** is carried out by several processes based upon two essentially different principles Fermentation is either allowed to go on in a quiescent liquid, comparatively rich in extract, or a liquid, composed of dilute alcohol and acetic acid with a small amount of nutriment, is exposed in drops to the action of an abundant supply of air

The best example of the former type of manufacture of wine-vinegar is the *Orleans* method It is carried out in horizontal open vessels partially filled with vinegar, receiving successive additions of bright wine until the vessel is four-fifths full A film of acetic acid bacteria will then form, which must not be broken The wine is soon converted into acetic acid, about one-half is then drawn off cautiously and fresh wine added, nearly as much as the vessel

can hold This proceeding is constantly repeated, the same vessel being used for several years The object of adding acetic acid is to prevent the growth of *Mycoderma* and other noxious fungi, for the same reason the fermentation is conducted at relatively low temperatures. To prevent foreign organisms finding a footing, Pasteur (as already stated) suggested sowing a young acetic film, while enlarging the surface of the liquid. In this way fermentation takes place more quickly At the same time, however, the risk is incurred that the formation of bouquet stuffs, characteristic of this kind of fermentation, may be diminished, and this applies also to more recent modifications of the method devised to accelerate the formation of acetic acid. Henneberg holds that the species just described are particularly active in this kind of fermentation A variety of liquors are used in the several countries of manufacture. Besides wine, we find cider, beer or a liquor prepared from barley, wheat, maize, and other fully fermented cereals, to each of which is added some vinegar rich in bacteria Alcohol is also added in some cases. Lastly, molasses and fermented wort from yeast factories are employed The product has more or less well-marked characteristics, according to the material used

When manufacturers began to understand the importance of atmospheric oxygen for acetic fermentation, various contrivances were introduced to supply abundant air during fermentation, and after much preliminary work an improved method, the "quick-vinegar process," was established by Schutzenbach in 1823. The principle underlying this method, as distinguished from the preceding one, is to insure the formation of acetic acid by the aid of an intense oxidation, carried out in fermenting vats of special construction. The vats are of a cylindric or slightly conical shape, 2 to 3 metres high, by 1 metre in diameter, fitted with two false bottoms with beech wood chips packed between them, to provide a large surface Below the undermost false bottom air-holes are bored in the wall of the vat. As the temperature rapidly rises in the vessels (28° to 38° C), a current of air is induced which accelerates the fermentation The cover is perforated or provided with tubes to act as outlets for residual air and gaseous fermentation products. The mash is poured upon the upper surface, whence it trickles through the chips, while the vinegar drips continuously from the lower false bottom and is drawn off If necessary, it is returned to the chips, with a further addition of mash to complete the fermentation. New methods have been developed on this basis, to utilise the raw materials to better advantage and obviate losses due to the evaporation of acetic acid This has been done by connecting several vats in series, allowing partially fermented mash from the first vessel to flow into a second vessel (with addition of mash if required), and so forth. Of late years, automatic systems have come into vogue The mash, containing a high percentage of acid, is

distributed from a common vessel through conduits in a slow stream into each vessel; this is done at definite intervals, by means of special compression and suction contrivances, adjustable to actual requirements.* The mash used in such a plant is made from water, alcohol, acetic acid, with a little nutrient. The organic liquids employed are such as beer, mashed cereals, yeast decoction; inorganic substances (regard being paid to the chemical composition of the water) include the primary phosphates of ammonium, potassium, and sodium, mixed approximately in the proportion of 7 : 2 : 1, with or without traces of ammonium, magnesium, and potassium sulphates. Larger or smaller doses of alcohol and acetic acid are added according to requirements. High-percentage mashes are considered to be the most rational, because the losses by evaporation are diminished by their use, and there is less risk of excessive oxidation or infection by mycoderma, etc.

It has been stated that Henneberg found peculiar species are active in these fermentations; they probably acquired their special characteristics by gradual adaptation to the medium. Materials for analysis are procured by transferring drops or shavings from the chips into beer or acidulated wort, or mash.

The use of pure seed cultures of selected and adapted ferments is widespread in both methods of vinegar manufacture. The pure culture, before being applied, is grown under conditions corresponding with those of the factory. When the Orleans method is employed, the bacteria are gradually acclimatised to the selected wine. A pure development of the chosen ferment can be best insured by pasteurising the wine in the plant. In the "quick-vinegar process" the culture is first acclimatised to large quantities of acid and alcohol, and minimal doses of organic foodstuffs. The culture is seeded in a vessel sterilised by steaming, and the vinegar run off is employed in the other vessels or added to the large mash-tun in the automatic system.

Acetic acid bacteria play an important part in the fermentation of beer, spirits, and wine. They do much harm, especially in wine, and if they once attain a strong development, the wine is irretrievably spoilt.

In *low-fermentation breweries* they usually do less mischief, as their growth requires a high temperature and an abundant supply of air. Thus they are readily suppressed in a well-arranged lager beer cellar. Hansen's experiments have shown that *Bact. aceti* and *Bact. Pasteurianum* are able to exist during the whole time of storage, whether the infection takes place at the beginning or end of the principal fermentation. In his experiments the contamination, however, did not manifest itself during the whole course of the fermentation either by the taste or by the smell of the beer.

* For details see Rothenbach, *Die Deutsche Essigindustrie*, Berlin; P. Hassack, *Gärungsessig*, Vienna, 1904; W. Hoffmann, *Gärungsessig*, Halle, 1915.

When the beer was bottled, and exposed to a higher temperature, the bacteria developed further, yet, when the bottles were well corked, the beer did not turn sour Just the same result was arrived at when the finished beer was infected. If, on the contrary, the bottles were badly corked, the growth turned the beer sour.

In *high-fermentation breweries*, on the other hand, where fermentation is carried on at higher temperatures, these bacteria are capable of doing much mischief, even before the beer leaves the brewery.

It is of practical interest to note that the species described by Hansen exert no influence on the colour or brightness of the beer, whilst most other bacteria cause turbidity

In *distilleries*, and more especially in *air-yeast factories*, acetic bacteria may occur in large quantities, as shown by numerous experiments made by the author They are most frequently accompanied by mycoderma species A careful control of the manufacturing process in this respect should never be omitted

While investigating the influence of acids, especially acetic acid, on *wine* yeasts, Lafar found that each of the different acids (malic, tartaric, lactic, acetic, etc) exerts a specific influence on the yeast, and not only on the proportionate amounts of alcohol and carbon dioxide produced, but also of glycerine, the acetic acid samples contained the smallest amount of glycerine and showed the weakest growth of yeast Contrary to the previously accepted view that even small amounts of acetic acid prevent alcoholic fermentation, Lafar found that the presence of 0 27 per cent had practically no influence on the rate of fermentation, the multiplying of cells, or the yield of alcohol and glycerine. In must, before neutralisation, the yeast cells were not impaired by an addition of 0 74 per cent ; and in neutralised must, after adding as much as 1 per cent. of acetic acid, 4 77 per cent by volume of alcohol was formed—*i e.*, 60 per cent. of the maximum yield Yeasts differ considerably, however, in their sensitiveness to the action of acetic acid Thus, a comparison of fifteen different wine yeasts showed that all were able to carry on fermentation in the presence of 0 8 per cent of acetic acid in a must that had previously been neutralised, whereas with 1 per cent. of acid only three were active With regard to the propagation of cells, yeasts react variously with the same amount of acetic acid Lafar also examined the influence of these cells on the ratio between the amount of alcohol produced and the number of yeast cells formed. He found, that in presence of 0·88 per cent. of acid, the amount of work done by a single cell was greater in the case of ten varieties, but smaller in two varieties, than in the presence of 0·78 per cent. Those yeasts which are active in presence of 1 per cent of acid, gave a smaller yield than in presence of 0·88 per cent.

According to Perold's researches on a number of wines in which acetic acid bacteria were allowed to grow freely, the various species concerned appear to differ considerably in the yield of acetic acid (from 6 to 9·6 per cent) Very actively acidifying species were generally met with in full-bodied wines, whereas the weakly acidifying ones occurred in milder wines. Acetic acid bacteria and acetic acid fermentation were even observed in certain wines containing up to 15 per cent by volume of alcohol

According to W Seifert, the nitric acid present in wines which have been diluted with water containing nitrates, is completely decomposed by the action of certain acetic bacteria

2. Lactic Acid Bacteria.

If the micro-organisms of milk are allowed to develop spontaneously at a temperature of 30°-35° C , the lactic acid organisms soon begin to ferment the lactose present (about 4 per cent.), and the acid produced protects the milk from putrefaction After a given quantity of acid has been formed it checks the activity of the bacteria, and the milk mould (*Oidium lactis*) begins to develop This oxidises a portion of the lactic acid, and thus enables the bacteria to restart their action The same effect is produced if the acid is neutralised, for instance with calcium carbonate, and thus the complete fermentation of the milk-sugar may be carried out. Simultaneously with the formation of lactic acid, casein, which forms the most important part of the albuminoid constituent of milk, separates out. Before souring, casein occurs as a calcium salt (100 casein to 1·55 CaO), and is present in a colloidal form. When milk is soured, the lime combines with lactic acid, liberating the casein, which is precipitated in a fine flocculent condition, causing the curdling of milk. In addition to this a greyish-yellow serum gradually separates out containing calcium lactate, lactose, albumen, etc

Other varieties of lactic acid may be cultivated by exposing a malt or other mash to a given temperature If the mash is maintained at 40° C. a pediococcus grows vigorously, if at 50° C , a short rod If a fraction of the liquor is transferred to another mash at the same temperature, the respective species is activated, and after a series of sub-cultures only the two species can be discovered in the respective mashes, by ordinary microscopical examination. It will be noticed that by the process described there can be no guarantee that a pure culture has been obtained, for in each case other bacteria survive, even if in an extremely weak condition, and, on the other hand, there is a possibility that more than one species, or variety, of lactic acid bacterium may develop at each temperature. Similarly in beer-wort and other liquids spontaneous lactic acid fermentation may occur This is also the case in the

souring of "Sauerkraut," the preparation of leaven, ensilage, etc.: the bacteria which develop in the several fermentations doubtless represent many different species.

The lactic acid generated by the fermentation of milk, an acid first definitely characterised by Scheele in 1780, corresponds approximately with the quantity of lactose that has disappeared. Only minute quantities of by-products are formed, as proved by the detailed researches of Kayser If the fermentation is continued for some time, many species will decompose part of the lactic acid originally found Kayser found that a pure cultivated species from cream grown in lactose-peptone-wort lost 0·26 g. of lactic acid per litre in eleven days If volatile fatty acids are formed, they tend to increase under such conditions at the expense of the lactic acid According to O Jensen, lactic acid itself may be converted into volatile fatty acids

Lactic acid formed by the spontaneous fermentation of milk is usually optically inactive—*i e*, it does not turn the plane of polarised light either to the right or to the left If however, the active bacteria are isolated in pure cultures and inoculated into sterile milk, species are developed, which produce a lactic acid turning the plane of polarised light to the right (dextro-rotatory bacteria), and others producing a lævo-rotatory acid. The dextro-rotatory species occur more frequently Thus the species of bacteria determines which acid shall be produced It appears, however, that with certain species of lactic acid organisms, the optical activity of the fermentation product of a given sugar depends upon the composition of the nutritive fluid, as shown especially by Kayser. The reaction of any one species depends upon the nature of the sugar supplied Thus the common *Bact. lactis acidi* (Leichmann) ferments dextrose, lactose, maltose, mannite, and raffinose Hueppe's *Bact acidi lactici* ferments saccharose, dextrose, lactose, and mannite. A few species thrive best when they have access to atmospheric oxygen, whilst others carry on the fermentation equally well, or even much better, in the absence of air They also vary in the rapidity with which acidification takes place at different temperatures Thus, the *Bact lactis acidi* has its optimum for the formation of acid at 32°-38° C, Hueppe's *Bact. acidi lactici* at 35°-42° C, others at 20°-22° C, and 40°-48° C. Broadly speaking, the largest amount of acid is generated at temperatures somewhat below the given optimum

In 1903, Herzog proved that *Bact acidi lactici* contains an enzyme which can be isolated from the living cell and is capable of producing lactic fermentation He treated a pure culture, ground with kieselguhr, with methyl alcohol, and subsequently with ether The mass was then dried, and the resulting white powder, which contained no living cells, proved capable of converting minute quantities of lactose into lactic acid

Buchner and Meisenheimer subsequently proved that if a culture of one of the species growing in a distillery mash, *Bact acidificans longissimus* (*Bact Delbrucki*, Leichmann), is treated with acetone, whereby it is killed, and the mass is then dried, a powder is obtained which can bring about a lactic acid fermentation in a sugar solution.

It may, therefore, be assumed that all bacteria of this group contain enzymes that can bring about fermentation independently of the living cell.

To cultivate lactic acid bacteria, a preparation of peptonised milk made by O Jensen may be used (*see* Chapter I)

In addition to the proper lactic acid organisms, there are a large number of bacteria, and, amongst them, some pathogenic forms, which develop this acid.

A characteristic feature of the true lactic organisms is that they do not form spores Most of them are non-motile Some of them flourish on artificial media where others will hardly grow The colour of the growth is usually white or yellowish Gelatine is only liquefied by a few species

In the course of years a large number of species have been described, but many of them should doubtless be considered as mere varieties of a few *types* The fact is that these bacteria are excessively liable to variation, and cultures seemingly possessing well-defined properties, when treated and grown otherwise frequently show marked variation Thus several species have been found to lose their power of forming lactic acid or one of the two isomeric acids, their power of fermenting given sugars has proved to be dependent on the sources of nitrogen in the nutrient liquid The faculty of forming slime may also vary very considerably in one and the same species, thus species not forming slime at temperatures favourable to their growth may become markedly slime-forming at low temperatures *

A remarkable *polymorphism* is met with in these bacteria, even within one and the same species An abstract of the classification proposed by Lohnis follows, giving a clear idea of the numerous varieties, to which we append some physiological characters peculiar to prominent species or types within each group

1 Short and Thick Rods

The short and thick rod form is considered to be the typical one; but there occur coccus-like forms and long threads, also chains resembling Streptococcus Optimum temp, 28°-42° C Most of these species will grow equally well whether air has access or not Optimum of milk-coagulation, 30°-40° C Generally produce much gas

(*B pneumoniæ Friedlander*) *B. acidi lactici Hueppe* (= *B ac lact. Grotenfelt*) *B.*

* Carbol and Richet, in their very extensive researches on lactic acid bacteria, showed that individual variation becomes the more pronounced the less favourable the vital conditions in which a culture is grown, even when antiseptics are present This seems to hold good generally

lactic aerogenes Escherich Transitions to *B ac. lact*, *B lactis acidi Marpmann* and *B lactis viscosus Adamez*, slime-forming

2 Streptococcus

Shape of cells very variable Often 0 5 μ broad, 0 5 to 1 μ long, oval, frequently tapering, lancet-shaped at one end, often paired or forming short chains of 4 to 6 links, also well-marked rods, together with spherical and flattened forms Particularly in broth, a tendency towards longitudinal growth of links and chains Oxygen exerts a more or less depressing influence on the growth, generally they grow more freely under anaerobic conditions Optimum temperature between 30° and 35° C Lactic acid nearly always dextro-rotatory Gas formation rare In spontaneously coagulating milk they are apt to get the upper hand because, in contrast to the first group, they are very active at temperatures below 30° C Non slime-forming races have been observed to pass into slime-forming.

Streptococcus Guntheri L et N, shown by Leichmann to be the most important agent of lactic acidification, the name was given by Lehmann and Neumann *Synonyms B. lactis acidi Leichmann, Micrococcus acidi lævolactici Leichm., B lacticus* and *Strepto. lacticus Kruse, B acidi paralactici Kozai, Lactococcus lactis Beijerinck*, and probably *B. lactis Lister* Further, *Streptoc acidi lact Grotenfelt, Micrococcus acidi paralactici Nencki and Sieber*, perhaps also Weigmann's *lactic acid bacteria Kiel I., II., III*, and Storch's *lactic acid bacteria*, partially, Troili Peterson's *Brachybacteria, Streptoc. thermophilus Jensen, Streptoc mesenterioides = Leuconostoc = Betacoccus Jensen, Streptoc. Kefir*

A large number of forms, which were described as species belonging to this group, are doubtless to be considered as mere varieties

Slime-forming forms —*Streptoc hollandicus* = Weigmann's ferment of "long Wei," *Bact lactis longi Tr Peterson, Bact lactis acidi Marpmann*, acid varieties of B. Gunther

3 Slender Rods

Breadth most frequently comprised between ½ and ¾ μ, length between 2 and 3 μ. Slender rod-shaped individuals, also short coccus-like forms and long threads Marked aversion to free oxygen Optimum temperature for several species, 40°-50° C Minimum at 25° or about 20° C Milk is, in general, coagulated somewhat slower than by *Streptococcus* Mostly lævo-lactic acid

B casei Freudenreich, B casei Leichmann, B (Thermobact) helveticum Jensen (B. casei E), B (Therm) lactis Jensen, Streptoc casei Jensen, B Listeri and *B Wortmanni Henneberg, Kefir bacillus Freudenreich, Lactobacillus fermentum Beijerinck, B caucasicum (Kern), B Delbrucki Leichm, B acidif long Lafar, Saccharobac Pastorianus v Laer, B bulgaric, B Mazun, B lebenis, Kefir-bact (B caucasicus, Dispora, Lactobac. caucasicus)* *

4 Micrococcus.

Cells spherical, mostly 0 8-1 6 μ Isolated, in pairs or in irregular clumps Optimum temp for most species 20°-30° C Some species grow equally well whether air has access or not, while others grow decidedly better with access of air Milk, if coagulated, is rendered minutely flocculent or compact The rennet enzyme is a concurrent cause of coagulation Liquefy gelatine partially Gas formation rare

(*M. pyogenes Rosenbach*) *M acidi lactis liquefaciens, M casei liquefaciens, Freudenreich's liquefying coccus, etc Gorini's acidifying cocci, M lactis acidi Marpmann, M. lactis acidi Leichmann, M candicans Flugge, Pediococcus acidi lactici Lindner, P cerevisiæ Balcke, Karphococcus pituitoparus Hohl* (causes ropiness), *M amarificans Conn, Leuconostoc mesenterioides*

Addendum. Sarcina.

We are indebted to Pasteur for the first important work on the subject of lactic acid bacteria In 1858 he described the species

* Stemberge attempted to classify these bacteria in two groups flocculent and non-flocculent, the former in malt extract will form flakes which readily settle down to the bottom, the liquid remaining almost bright, whereas those of the latter group remain suspended in the slowly clarifying liquid There exist, however, transitional varieties

which appears when *milk* spontaneously ferments. In his *Études sur la bière* he depicts certain bacteria growing in *wort or beer* in which lactic fermentation has begun; he describes them as short rods slightly constricted in the middle, and commonly occurring singly, rarely united in chains. In 1877 Lister prepared a pure culture of a lactic acid bacterium from *sour milk*, which he called *Bacterium lactis*.

In 1884, Hueppe found a bacterium in a spontaneous lactic acid fermentation which converts lactose and other sugars into lactic acid with the simultaneous formation of carbon dioxide (*Bact. acidi lactici*). It consists of short, plump, motionless cells, the length of which exceeds their breadth by at least one-half; they are united chiefly in pairs, and seldom in groups of four. In gelatine plates they form whitish colonies; those below the surface are stellate, uniformly dark, and sharply outlined; on the surface they appear as flat, white, glistening nodules, resembling porcelain, surrounded by clear outer zones. Atmospheric oxygen is necessary for fermentation with this species.

In recent times a large number of species of lactic acid bacteria have been found in *milk*. Marpmann, in 1886, described five species embracing both coccus and longer and shorter rods, and showed that the whole series is capable of producing a slight formation of alcohol.

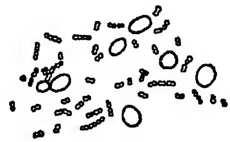


Fig. 22.—Lactic acid bacteria (after Pasteur).—In order to give an idea of the size of the bacteria, some yeast cells are figured among them.

Hueppe and Grotenfelt have since described new species, of which Grotenfelt's *Streptococcus acidi lactici* appears to be identical with the *Bact. lactis acidi* described by Leichmann.

Adametz and Freudenreich have isolated species from Emmenthaler cheese (*Bac. casei*) which are for the most part facultative anaerobes.

Leichmann has thrown new light on the conditions present during the spontaneous souring of milk. He found that a single species or type strongly preponderated, and named it *Bact. lactis acidi* (*Streptococcus lacticus—Str. Guntheri* L. and N.). He described it as consisting of short motionless rods about one and a half times as long as they are broad, sometimes present in pairs, sometimes in chains (the latter particularly when cultivated in sugar-broth). On gelatine plates, the immersed colonies consist of round discs, white or pale yellowish-brown, at first transparent, afterwards opaque. The surface colonies develop with extraordinary difficulty; they are transparent, and have a somewhat irregular edge. This species excites fermentation even in complete absence of air; in presence of a full supply of air, fermentation is restricted. Both Leichmann and Weigmann consider this species to be the regular

means of spontaneously curdling milk, and explain the process of souring as follows —Hueppe's *Bact acidi lactici*, and other species of the aerogenic group, remain in the upper layers of the milk, to satisfy their great demand for air and acidify them; whilst the facultative anaerobe, *Bact lactis acidi*, develops in the lower layers, and acidifies them This species ferments lactose and dextrose together with maltose, raffinose, and mannitol It forms dextro lactic acid, and produces no evolution of gas in sterilised milk.

In spontaneously soured milk, Leichmann discovered another species of frequent occurrence, which has great similarities with the above, but may be distinguished by its production of lævo-lactic acid, and by the evolution of gas (*Micrococcus acidi lævo lactici*) He also isolated a species which thrives best at 41°-52° C., develops lævo-lactic acid, and forms thin rods of varied length. On agar it forms root-like, branching colonies The great series of interesting varieties cultivated in a pure state by Weigmann deserves special attention Their appearance at first resembles that of Leichmann's *Bact lactis acidi* Biologically, however, they show important differences, and several have found widespread industrial application An organism generally occurring in milk pasteurised at relatively low temperature is the *Streptococcus thermophilus*—described by O Jensen—which makes its appearance in Emmenthal cheese when the temperature in the press is falling slowly from 50° to 35° C It presents the same forms as the other streptococci, and grows freely at 40°-45° C In milk it forms short chains at 45° C., in agar long chains at the same temperature. It forms large quantities of acid On green vegetable matter and juicy roots, particularly on beets, there occur several *streptococcus* forms grouped together by Jensen under the name of *Betacoccus* (including *Leuconostoc* = *Strpt mesenterioides*, to be described in the sequel) They are subject to great variation in form, in their reaction on the sugars and in their power of forming slime. Other species have been described by Kayser, Marpmann, Conn, Kozai, Beijerinck, etc In addition to these typical lactic acid bacteria, other species occur in the souring of milk which possess a curdling and a peptonising enzyme

The ripening of **cheese**, which consists in the conversion of casein (paracasein) into simpler albuminoids, and the breaking down of the latter, is chiefly a biological process As is well known, the curd may be separated from the milk by the addition of rennet,*

* Rennet is an enzyme which decomposes casein into paracasein and whey albumen. It is secreted in special glands of the stomach of various animals (*e g*, the ruminants); calves' stomachs are used for the preparation of rennet In the vegetable kingdom this enzyme is widely distributed It is found, for example, in *Pinguicula*, *Ficus carica*, *Galium verum*, in the calyx of the artichoke (*Cynara scolymus*), and in many bacteria. In 1892, Conn isolated an enzyme resembling rennet from bacteria which were isolated from cream, and completely liquefied gelatine They produced the enzyme most rapidly and freely at about 20° C He isolated it from the filtrate of a ten-day-old milk culture.

whereby it remains sweet, or by the application of lactic acid (sour milk) The gradual decomposition of the curd is due to a slight extent to the action of pepsin contained in rennet, as well as to an enzyme (*Galactase*, Babcock and Russell) The action is, however, mainly due to the rich flora of micro-organisms embedded in the cheese

The basis of bacteriological work on cheese was laid by Cohn, Duclaux, and Benecke, and their researches have been extended by Adametz, Rodella, Burri, Freudenreich, Weigmann, O Jensen, Gorini, Barthel, and Harding The main lines of this development must be regarded, according to these workers, as a modification of the albuminoids of the curd brought about during the first short period by *peptonising bacteria*—bacteria that had been active in the milk Amongst these must be especially mentioned *Micrococcus casei liquefaciens*, which occur in great numbers. It multiplies at lower temperatures than the true lactic acid bacteria, and is therefore found in large numbers in the cooled milk, and is thus transferred to the cheese It coagulates milk in 24 hours at 35° C, ferments lactose, and produces volatile acids, especially acetic acid Its growth is, however, soon arrested by *the true lactic acid bacteria*, and, in particular, by the development of *Bact lactis acidi* (*Streptococcus lacticus*), which causes a vigorous formation of acid

The bacteria belonging to this group were shown by Bartel to possess also a considerable power of splitting up casein, in consequence of which, when grown at relatively low temperature, they exert an appreciable influence on the ripening of cheese The lactic acid gradually combines with the lime and phosphates of the casein or it is removed by acid-consuming fungi or alkalising bacteria, thus enabling peptonising or casease bacteria, more particularly the aroma-forming species, to commence operations. Their development is, however, regulated by the lactic bacteria, which prevent the casein being broken down too rapidly

An essential *rôle* in the ripening process devolves upon certain rod-shaped species of lactic bacteria (*B casei E* in Emmenthaler cheese), particularly at relatively high temperatures, in breaking down albumoses and peptones to amino-acids, at which stage the development in hard cheeses is frequently arrested At the same time volatile acids are formed acetic and propionic acid. In Emmenthaler cheese O Jensen discovered special propionic acid bacteria, the action of which is supposed to account for the

The filtrate was acidified with 0 1 per cent of sulphuric acid, and then mixed with an excess of salt A white foam separated out, which contained the comparatively pure enzyme The dry foam formed a white powder This enzyme also occurs in the ubiquitous putrefactive bacterium, *Bacillus vulgaris* (*Prot vulg*) It further occurs in *Bact prodigiosum* (the bacterium of the Bleeding Host), and also in *Bact coli communis*, which is always found in the intestines of men and animals, as well as in many of the "potato bacilli" Finally, it has been detected in torula species (*Lactomyces*), in different species of moulds (*e g*, *Aspergillus*, *Monilia*), and in certain yeast species

formation of "eyes" in this cheese According to Wolf, however, it is quite possible that under special conditions, particularly during the formation of lactates (calcium lactate), the common lactic acid bacteria may produce propionic acid in cheese Clark showed the formation of "eyes" to be particularly favoured by a slow fermentation of the cheese, while in a quick fermentation the carbon dioxide is distributed throughout numerous small cavities

According to Gorini, in hard cheeses, in the more advanced stages of fermentation, a typical growth appears of special cocci of various types, described by him as acid-rennet-forming The casein is transformed, with acid reaction, by a particular rennet enzyme, and afterwards successively and completely broken down by their proteolytic enzymes, at temperatures between 20° and 37° C, especially at the lower temperature

Among the specific casein-digesting bacteria must be mentioned the so-called *Tyrothrix* species (Duclaux, 1878), which appears to be closely allied to the hay bacillus.

In the various sorts of cheese there doubtless occur very different species and types of casein-digesting bacteria, whose well-known tendency to variation appears, in their co-ordinated action, to determine in a large measure the final result of the fermentation

In *soft cheeses* certain species of bacteria are found to predominate in succession, while at the same time several mould fungi help to impart to these cheeses their peculiar characteristics. The decomposition of albuminoids in soft cheeses is usually carried further, part of the amino-acids being decomposed, with formation of ammonia and other pungent products Concurrently with this partial putrefactive process a splitting up of fats takes place, contributing to the piquant flavour characteristic of these cheeses. Among the bacteria a specific part is played by the anaerobic spore-forming species, including certain butyric acid bacteria. As an example may be given *Paraplectrum foetidum*, discovered by Weigmann in Limburg cheese, in milk it appears as thick rods, which at 30°-40° C assume the shape of a club and rapidly form spores, twice as long as they are broad The action of mould fungi partly consists in directly consuming some of the acid, and partly in helping to decompose the casein, forming ammonia and neutralising free acid, so paving the way for the active bacteria. Finally, they are also able to decompose milk fat and set free the volatile fatty acids Among the moulds we may mention the *Mucor*, *Penicillium* and *Dematium* species found in Norwegian "Gammelost" (old cheese), in Camembert and Brie a white *Penicillium* (*P candidum Roger*, *P album*), certain *Oidium* forms are also believed by some authorities to play a part in the fermentation of these two cheeses. The peculiar taste of Roquefort cheese is chiefly due to the growth of *Penicillium Roqueforti* According to Stennart this

species is generally found to develop in Gorgonzola, Stilton, Wensleydale, and blue Dorset cheeses

The abnormal characters of **milk** and milk products must to an equal degree be attributed to micro-organisms * Thus, for instance, the bacteria introduced into milk from a diseased udder, and consequent changes in the character of the milk, are accompanied, not only by a very great increase in the bacterial contents, but also by the presence of characteristic pus cells in the milk.

"Soapy" milk, having a decided soapy taste and producing a strong lather, owes these properties to the presence of *Bact lactis saponacei*, a short rod which forms slimy colonies on ordinary nutritive gelatine, turning rusty yellow on the surface. Other species may also produce this fault

Bitter milk may be the result of using certain food-stuffs, but may also be produced by bacteria, as was shown by Pasteur, Duclaux, Löffler, Weigmann, and others In practice, micrococci which liquefy gelatine and certain varieties of aerobic lactic acid bacteria appear to have this effect Certain *Torula* yeasts may grow in milk and make it bitter (Calloghan and Harrison) "Ropy" milk has a marked slimy character, and can be drawn out into threads This is due either to the enormously swollen membrane of certain bacteria, or to the formation of slimy albuminoid bodies. The active micro-organisms are either varieties of lactic acid bacteria, or more usually bacteria that digest albumen A widely distributed species is *Bact lactis viscosus*, described by Adametz, which gradually converts the viscid milk into a substance resembling honey Another widely distributed and active species is *Micrococcus Freudenreichii*, described by Guillebeau, which liquefies gelatine The lactic acid bacterium, *Streptococcus hollandicus* Hueppe, described by Weigmann, Goethart, Boekhout, and others, is of particular interest it occurs in Dutch "lange Wei" (a ropy cream used in the manufacture of Edam cheese). This organism is a facultative anaerobe, and has its optimum at 21°-22° C Like many other varieties it easily loses its property of forming slime A similar variety was found by G Troili-Petersson in the Swedish "tatmjolk" (thick milk), and described under the name of *Bact lactis longi* It is believed that this species constructs slime from lactose It has its optimum below 20° C Probably such bacteria are present on certain plants that are steeped in the milk, such as *Pinguicula* and *Drosera*

By the action of micro-organisms milk may assume a blue, red or yellow colour The blue coloration of milk depends upon the growth of certain species of bacteria, the presence of which was proved by Fuchs as early as 1841 Hueppe was the first to prepare

* It is generally accepted that milk may act as a carrier of many dangerous disease germs, typhoid epidemics in particular appear to spread in this way Tubercle bacilli, capable of development, have frequently been found in raw milk

a pure culture of one of these species, and he described it under the name *Bacillus cyanogenes* (*Bact. syncyaneum*), which occurs as a short motile rod. In the case of this, the most widely distributed species, the colour appears first on the surface of the raw milk, and afterwards penetrates to the lower layers. The colouring matter is derived from the albuminoids, and may appear in the absence of sugar. A number of the water bacteria also have the power of imparting a blue colour to milk. The reddish colour which milk occasionally assumes is also due in certain cases to bacteria. Only a few examples are quoted in the technical literature. Hueppe found a *Bact. lactis erythrogenes* in red milk, which is described by Grotenfelt as a short rod coagulating milk, and producing a red colour on gelatine plates. Menge found a *Sarcina rosea* in red milk, which also forms red colonies on gelatine, and a few other species with similar properties have been detected. In yellow milk a *Bact. synxanthum* has been observed and described by Schroter.

The taints observed in **butter** must also be chiefly attributed to micro-organisms, and the technical literature strongly impresses the fact that a great development of such harmful species is frequently caused by want of cleanliness, or by the wrong souring of the milk. The rancidity of butter, which is due to the presence of butyric acid and ethyl butyrate, is caused by the action of light and air. According to O. Jensen this phenomenon is due to the presence of aerobic fungi, which cause the decomposition of fat, in particular *Cladosporium butyri* and *Oidium lactis*. It may further be due to the presence of two bacteria universally found in water, *Bact. fluorescens liquefaciens* and, less frequently, *Bact. prodigiosum*. The action of light may also produce the tallowy taste, but Storch has isolated a rod-shaped lactic acid bacterium which can produce the same effect. The "yeast" taste is supposed by Rosengrén to be due to the fact that at too high a temperature of acidification, certain rod-bacteria of the group *B. acidi lactici* are apt to grow freely and hence to suppress the streptococci; the increase if acidity thus produced will further the multiplication of the yeast cells present and, consequently, give rise to a taste like that of sour bread or beer. A turnip flavour and a rotten-sweet flavour in butter, studied by C. O. Jensen, proved to be derived from a special species, *Bact. foetidum lactis*. Weigmann subsequently observed similar bacteria. A whole series of other irregularities in the character of butter are also accompanied by the growth of specific micro-organisms, and it is reasonable to assume that they must be the originators of these taints.

One of the most pronounced faults with **cheese** is "blowing": it is due to the presence of great masses of fermentation bacteria causing an excessive development of gas; the species *Bact. Schafferi*, belonging to the *Bact. coli* group, is frequently responsible for

this defect The same fault may be produced in cheese by species originating from diseased udders Yeasts may produce a strong evolution of gas and certain varieties of *aerogenes* species may also bring about vigorous fermentation with production of gas A suitable degree of souring with lactic acid bacteria appears to be a certain means of preventing these mishaps

The blue flecks which appear in certain kinds of cheese may be produced in some cases, according to Beijerinck, by *Bact. cyanofuscum*, which is derived from water Black flecks may be caused by growths of moulds, such as *Cladosporium* and *Fumago* Rusty specks, according to Connell, Harding, and other American observers, are caused by a definite species, *Bact rudense* In the same way a reddish colour is produced by red moulds, micrococci, etc

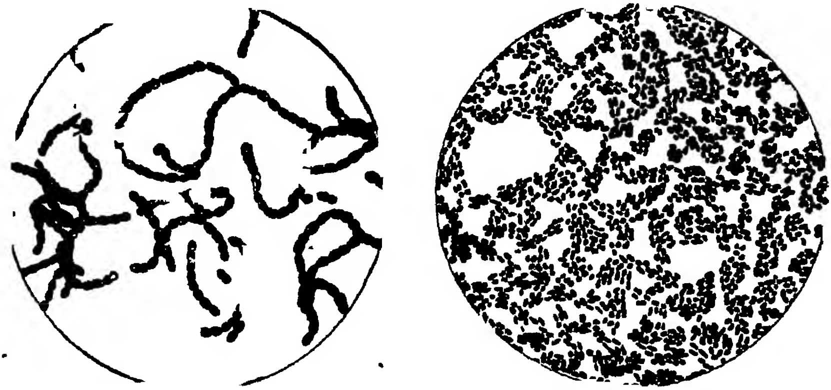
Lastly, a fault must be mentioned which may occur in all kinds of cheese, the bitter taste, which is caused by certain bacteria, as, for instance, by *Micrococcus casei amari*, described by Freudenreich, and also by a species occurring in bitter milk, and even by certain moulds and *Torulæ*

Since 1890 methodically selected species of bacteria have been applied in dairies, to bring about a regular and certain souring of the cream used in the manufacture of butter, and to avoid any taint in butter The progress made in this field is associated with the researches of Storch, Weigmann, Quist (in the author's laboratory), and others The pure culture selected is added to skim milk previously heated to about 90° C, and the culture is allowed to develop at about 25° C After standing 24 hours, this "starter" is fit for use In order to render the cream which is to receive the culture as free from germs as circumstances permit, it is pasteurised at about 85° C, and then quickly cooled In the course of ten hours or so, the starter is allowed to develop in cream at about 16° C It is then cooled below 10° C, and churning is begun

Among the forms isolated by Storch of Copenhagen (1890) from butter, sour cream, and butter-milk, the coccus form of the group *Streptococcus lacticus* (*Str cremoris Jensen*) seems to be most frequent and best suited to sour the cream It occurs in a large number of varieties, which, according to their main characteristics may be classed in two groups—one including those which give a specially pure and mildly sour taste and a fine aroma, and another embracing those which yield a product possessing great keeping powers Morphologically the races are distinguished from each other by the fact that some are linked in chains, others are not (Fig 23), the latter are of the most frequent occurrence, and are most widely distributed These forms bear a certain resemblance to Pasteur's "ferment lactique" The species represented in Fig 23, *B*, was isolated by Storch from a sample of butter having a pure and full aroma It forms small globular colonies in gelatine of a pure white colour and smooth surface In milk and whey it

occurs in oval or globular forms These lactic acid bacteria display fermentative activity even at 20° C At 28° C milk is turned sour within eight to nine hours

Many species have been isolated by Weigmann and introduced into practice A species which has been very successfully applied at several places was prepared in the author's laboratory It occurs both as micrococcus and in other forms, according to the different nutrient media in which it is cultivated. On gelatine it forms small, circular, slowly-growing colonies of a whitish-yellow colour. In stab-cultures spherical colonies arise throughout the puncture-channel, and in streak-cultures this organism forms a continuous streak with wavy borders. It was prepared from a sample of butter of remarkable aroma and durability



A *B*

Fig 23 —Lactic acid bacteria (after Storch)

Pure cultures of lactic acid bacteria have also been applied in cheese factories to regulate the ripening of cheese They are always added to raw milk, as it is of importance that other species of bacteria from the milk which play a part in the process should not be suppressed Cultures of other bacteria and of moulds have been applied in the preparation of cheeses of pronounced character—*e g*, Roquefort, Camembert, etc

As the mash in **distilleries** is not allowed to exceed a temperature of 70° C, in order that the diastase may be preserved, many of the germs adhering to the raw materials are not killed, but are capable of developing during fermentation, and thus they may not only utilise the nutritive substances, but also disturb the desired alcoholic fermentation, in the latter respect, butyric acid bacteria are specially dreaded With a view of preventing too strong a development of germs injurious to the yeast, various acids have been added direct to the mash, or else a lactic acid fermentation is previously

carried out in a concentrated and saccharified mash (about one-tenth of the principal mash), which is kept at a temperature of 50°-55° C., till it shows about 2¼° of acidity,* corresponding to about 1 per cent. of lactic acid. At this temperature the selected species of lactic acid bacteria develop, whereas it is too high for the majority of bacteria. An excellent means for maintaining the mash at this temperature is the acid chamber introduced by Kruis into distilleries, a small and well-isolated space in which the air maintains a constant temperature; in which the mash to be soured is placed and allowed to stand at rest as soon as it is cooled to room temperature. The mash, after acidification, is heated up to 70°-75° C., whereby part of the lactic acid bacteria are killed. After subsequently cooling to about 20° C., the yeast is added. The yeast is not affected by this amount of lactic acid. After it has developed sufficiently, the mixture is employed for pitching the principal mash. If the acidification of the yeast mash is carried out in the proper manner, only the useful lactic ferments will develop during fermentation. It is true that the other bacteria will not all be killed (more particularly sporulating species are apt to survive), but in a regular, active fermentation they are usually unable to multiply in the chief mash. About one-tenth of the acidified mash must be placed on one side before yeast is added, and used to start the souring of the succeeding acid mash.

The acid thus introduced into the principal mash, together with the surviving lactic acid bacteria, thus act as disinfectants, besides exercising an influence on the yeast cells, both directly and by reacting on the nutritive substances.†

In air-yeast manufacture the whole of the wort is submitted to direct acidification.

The lactic acid bacteria occurring in the mash can be distinguished in many ways from those occurring in milk. Zopf was the first to prepare and investigate a culture of a species belonging to this class, isolated from a mash obtained from dry malt and water at 50° C., according to Delbruck's process (1881), following up an observation of Delbruck's that at this temperature

* *i.e.*, 2½ c.c. of normal caustic soda solution are required to neutralise 20 c.c. of mash.

† With regard to the effect produced by lactic acid bacteria on *alcoholic yeasts* during simultaneous growth on a nutrient medium, it was established by Johannessohn and Steinberge that those lactic ferments which produce large quantities of volatile acids are apt to act deleteriously on yeast with respect to its development or fermentative power, or else they may cause agglutination of the cells. A kind of symbiosis was found to occur between some of these species and yeast, as they produced considerably larger quantities of volatile acids during the development of the yeast than otherwise. Further, Kayser and Delaval proved symbiotic inter-relations exist between certain lactic bacteria (from infusions, pickled cabbage, and from a bakery), and yeasts incapable of fermenting maltose and lactose (*S. Marxianus* and other species), which sugars, dissolved in the nutrient liquid, were entirely decomposed. A distinct reaction for alcohol was observed.

a vigorous lactic souring took place A growth of threads, rods and cocci was observed

Pediococcus acidi lactici, examined by Lindner, gives a strong acid reaction when cultivated in a neutral malt-extract solution at 41° C. Both in a solution of this kind and in a hay decoction which has not been sterilised, this bacterium develops so vigorously that, according to Lindner, all other organisms are suppressed at this temperature It has been proved chemically that the acid, which is abundantly produced, consists for the most part of lactic acid When a malt mash or malt-rye mash is maintained at 41° C, the *Pediococcus* develops vigorously, and the rod-shaped lactic acid bacteria are suppressed According to Henneberg the optimum for the formation of acid is 38° C The optimum for growth on beer and wort agar lies between 36° and 40° C In a neutral malt-extract solution the *Pediococcus* is killed after five minutes' exposure to 62° C On gelatine it does not thrive well, it is only in stab-cultures in neutral malt-extract gelatine that very vigorous white colonies are formed below the surface This species appears, on the whole, to thrive better when air is excluded

In 1893, Kruis and Rayman isolated a vigorous lactic acid bacterium from yeast mash consisting of long and short rods, which produced 0 9 per cent of lactic acid at 40° C in a clear malt wort It is of special interest to note that Kruis and Rayman in studying this species proved, for the first time, that lactic acid bacteria are capable of forming volatile fatty acids

Lafar isolated from the sour yeast mash a species which he named *Bact acidificans longissimum*, and since 1894 it has been applied in practice for souring yeast mash It ferments saccharose, galactose, dextrose, lævulose, and maltose, but not lactose, and it occurs both in short rods and in very long filaments A short time afterwards, Leichmann described a bacterium occurring under similar conditions, *Bact Delbrucki*, which is believed to be identical with Lafar's species It shows great resemblance to Leichmann's *Bact. lactis acidi*, and both species produce lævo-lactic acid It cannot, however, like the latter, ferment lactose In a lactose broth it produces no acid, and grows with difficulty, whereas in grape-sugar broth or maltose broth, as well as in sweet wort, it grows vigorously According to Henneberg this species has its optimum for acid production at 46°-47° C In the mash it forms up to 1·79 per cent. of lactic acid. The amount of acid is reduced with free access of air. Its optimum for growth lies between 40°-48° C. In the mash, it occurs with both short and long cells, single or grouped two and three together. On solid substrata it forms small, flat, clear colonies.

Henneberg isolated a number of other species of lactic acid organisms from mash and pressed yeast, which he described as "wild," some of which may produce direct damage in the in-

dustry, if care is not taken to secure a vigorous yeast fermentation, for they not only carry on the production of acid throughout the fermentation, but at the same time form volatile acids, especially acetic acid, which damages the yeast and reduces the output of alcohol Other members of this group appear to be harmless.

All the species examined grow and produce acid in presence of yeast at 27 5°-30° C They are killed at the temperature of saccharification, so that infection can only be effected at a later stage. Amongst dangerous kinds may be named *Bact. Hayducki*, which occurs in mash in small short cells, mostly single, and forms round white colonies on gelatine (its optimum for acidification is first at 45°-46° C , later at 33°-35° C), and *Bact. Buchneri* with similar cells in the mash, and white or yellowish colonies on gelatine (optimum for acidification first 39°-40°, and afterwards 23°-30° C)

In *flocculent* pressed yeast Beijerinck, Henneberg, and others discovered some special types of lactic acid bacteria, to the action of which they attributed this property of the yeast Among these bacteria may be mentioned Beijerinck's *Lactococcus agglutinans*, which on wort-agar at 23° C produces small, compact, moderately dry colonies, and will grow luxuriantly in closed bottles containing wort at 30° C , further, *Lactobacillus conglomeratus*, which on wort-agar at 40° C. occurs in abnormal twisted forms together with cocci and filaments Beijerinck found it grew freely at 33° C in wort inoculated with putrescent pressed yeast in a closed bottle Henneberg describes *B Listeri*, which consists of short and long rods with blunt ends, single or united in chains or clots , maximum temperature of acidification in mash is 42°-45° C. , optimum 34° C ; and *B. Matthesi*, small cells, forming short or irregularly curved longer chains Optimum for acidification of mash 27°-34° C., afterwards 23° C At 42° C no growth This species occurs frequently on malt and grows readily at 25°-30° C. on bruised malt soaked in water

There appears to be no doubt that the lactic organisms occurring in the mash have a tendency to variation, and that not a few of the numerous species described must be regarded as sub-species of a certain parent form.

Thus Beijerinck takes as the parent form of Leichmann's species, *Bact Delbrucki*, a very vigorous acid producer, *Lactobacillus fermentum*, a young culture of which prepared at 37° C. for 36 hours in an acid mash and then transferred to wort jelly (gelatine agar), forms very small transparent colonies consisting of non-motile bacilli of varying length together with micrococci In wort it forms filaments varying in length The smaller the amount of oxygen present, the more extended are the cells Its optimum for the production of acid is 41°-42° C , and at 50° C it ceases to produce acid. According to Beijerinck, this parent form

may be converted into *Bact Delbrucki* through continued subculturing with free access of air at 48° C (well above its optimum for acid production), and then transferring to wort jelly at 37° C.

In the distillery, Lafar, Leichmann and the author (in 1896), and more recently Kusserow and others, have introduced into practice pure cultures of predominant species occurring in normal sour mash (*Bact acidificans longissimum, Bact Delbrucki*), with the object of regulating the souring of the mash By the proper application of such cultures in distilleries and yeast factories, and at the same time securing a uniform temperature of acidification throughout the whole mash, it is possible to absolutely prevent the development of foreign and harmful bacteria The mash used should have a restricted supply of air, if an abundant quantity of air is injected, the formation of acid will be checked

In the **brewing industry** the lactic acid fermentation takes place in malting, and in particular in mashing During the **germination of barley,** lactic acid bacteria are of essential importance to the progressive development of the diastatic and peptic enzymes, by which a rational carrying out of the germination process is conditioned The acid formed in the **mash** is for the most part lactic acid, partially produced by spontaneously occurring bacteria. It exerts a favourable influence on the diastase and peptase working in the mash If the water contains a comparatively large amount of carbonates, the free lactic acid in the mash will be neutralised, which will have an unfavourable influence on the palatable quality of the beer and its sensitiveness to cold This can be prevented by reducing the amount of carbonates in the water, or by increasing the acidity by addition of selected cultures of lactic acid bacteria Wahl and Braun worked specially in the latter direction. Sorensen's very extensive investigations have served to elucidate these processes in general, and to determine the degree of acidity (hydrogen ion concentration) which gives the most favourable conditions for the action of enzymes The use of pure cultures of bacteria has been found to give excellent results whenever due allowance is made for the character of the water and brewing materials

Some lactic acid is also formed during fermentation. Lactic acid is formed in large quantity in Belgian beers prepared by "spontaneous fermentation," imparting a sharp taste "Weissbier" owes its refreshing taste to a vigorous lactic acid fermentation In modern low-fermentation breweries attempts are made to get rid, not only of lactic acid bacteria, but also of bacteria of all kinds from the fermentation

The Saccharobacillus Pastorianus described by van Laer, which occurs in the form of rods of different lengths, produces a characteristic disease ("tourne") in weakly hopped beer, which manifests

itself as follows —The liquid gradually loses its brightness, and when it is agitated filaments of a silky lustre rise from the bottom, and the beer assumes a disagreeable odour and taste According to Fellowes, this species is also found in English beer. It does not always react on the beer, probably on account of the larger amount of hops In cultures, the bacillus develops either in the presence or absence of free oxygen In nutrient liquids it ferments carbohydrates, and amongst them the saccharoses, without previously inverting them Amongst its fermentation products, lactic acid, acetic acid, and alcohol predominate The acids produced cause the precipitation of nitrogenous compounds in the liquid, and these, mixed with the bacilli, produce a cloudiness, consisting of lustrous filaments The nutritive mixture best suited to this bacterium is an extract of malt mixed with agar and a small quantity of alcohol, or, still better, neutral or slightly alkaline sweet wort.

If this bacterium is exposed to a temperature of 55°-60° C in beer, it is soon killed Henneberg has closely investigated both this species and two other lactic acid bacteria occurring in beer which cause the same disease—*Saccharobacillus Past*, var *berolinensis* and *Bac. Lindneri* These all occur as thin and comparatively long bacilli, either straight or curved, and usually cluster together In hanging drops they form very long threads. From a physiological point of view they behave quite differently *Saccharobacillus Past* gives the most vigorous formation of acid in arabinose and trehalose, weaker in saccharose, maltose, dextrose, lævulose, and galactose Its optimum for acid formation lies between 24° and 33° C The variety *berolinensis* gives a weaker yield of acid, and none at all in raffinose and trehalose Its optimum for acid formation is at 20°-24° C. According to Henneberg it is this species in particular which grows in Berlin " Weissbier," and imparts to it its peculiar character Other varieties of lactic acid bacteria occur, however, in this kind of beer.

Bact Lindneri produces acid in maltose, and to a small degree in dextrose The optimum for acid formation is at 17°-18° C. This species or variety frequently occurs in ordinary lager beer, and influences its flavour and aroma, without, however, producing any considerable amount of acid. It may occur in the form of long cells in lager beer

Schonfeld observed a species in various high-fermentation beers, which looks like the bacterium of Berlin Weissbier, and gives to these beers a slight lactic acid flavour, and may make the beer turbid.

Very extensive researches on the diseases of **wine**—by Pasteur, Muller-Thurgau, Osterwalder, Wortmann, Kayser, Monceau, Vergnette-Lamotte, A. Koch, Mazé, Pacottet, Laborde, Seibert, Kulisch, Schukow, and several others—have shown that bacteria forming lactic acid play a prominent part; it was particularly Muller-

Thurgau and Osterwalder who, through methodical investigations into the effects produced by pure growths, established the work on a solid foundation, and insured reliable results The following is a summary of the various diseases of wine, based mainly on the exhaustive work of Muller-Thurgau

The disease bacteria may occur both in the course of the alcoholic fermentation and after the completion of the primary fermentation; the latter remark applies chiefly to lactic acid bacteria In later stages of development those species which cause red wines to assume a bitter taste are apt to develop. So also the particular group of bacteria which bring about a decrease of acidity mostly occur after completion of fermentation

Staleness and the vinegar taint are known to be produced by mycoderma vini and acetic acid bacteria respectively

The lactic acid taint, as caused by lactic bacteria, occurs chiefly in fruit wines and mild grape wines Besides lactic acid, they contain a good deal of acetic acid This disease often appears in the course of alcoholic fermentation itself, more particularly if the temperature rises The bacteria concerned are the same as those which produce **mannite** fermentation, when the liquid contains lævulose, from which they produce mannite as well as lactic and acetic acids Thus, according to Muller-Thurgau, the characteristic taste may occur without mannite fermentation, when no lævulose is present

Toughness and ropiness (*la graisse*) is a peculiar disease, which manifests itself by an oily consistency of the wine; the latter, when poured out, appears turbid and ropy, and flows soundlessly into the glass. The phenomenon was thoroughly studied by Kayser and Monceau, who proved the growth of particular anaërobic bacteria to be the cause of the disease short, rounded rods of differing dimensions, single or in short or long chains According to Kayser and Monceau, they form mannite and lactic acid. Associated with them are always to be found various aërobic species—bacteria, cocci, sarcina—which are considered to aggravate the disease. The causes of the disease have not been entirely solved, but at all events it is certain that the composition of the wine, and more particularly the amount of alcohol and free acid, is of great importance The so-called *Bockser*, characterised by the wine containing hydro-sulphuric acid, seems always to be due to the action of micro-organisms, especially yeast, when free sulphur is present in the fermenting liquid, yet the latter condition is not to be taken as absolute **Turned** wines (*vins tournés, pousse*) were first closely examined by Pasteur (French red wines), later by Duclaux and other French *savants*, and also by Muller-Thurgau. This disease first appears as a silk-like turbidity (*ondes soyeuses*) caused by bacteria, afterwards, especially if air has access, the wine changes its colour, secreting a red colouring matter, and

at the same time the smell and taste undergo a change The bacteria concerned appear to be principally facultative anaerobic; they take the form of threads, rods, and cocci. Muller-Thurgau pointed out that this phenomenon is not to be confounded with the "repeated turbidity" caused by yeast, bacteria, and secretions.

The so-called *Mauseln*, characterised by a most unpleasant smell and taste (like that of acetamide), is traceable to the action of particular bacteria, especially (as shown by Muller-Thurgau, who worked with pure cultures) the *B mannitopœum* described below, which brings about these effects by decomposing lævulose, dextrose, and saccharose Finally, we return to the **bitterness of red wines,** as accurately described by Vergnette-Lamotte (Pasteur's *Études sur le vin*) The wine first assumes a stale sweetish taste and peculiar smell, quickly supplanted by an increasing bitter taste, the colour changing at the same time Concurrently, the amount of glycerine is found to decrease According to Muller-Thurgau, the disease is caused by facultative anaerobic bacteria, which chiefly occur in rod form, sometimes in threads Wortmann holds that *Botrytis* is associated with the disease, as this organism gives rise to chemical changes in the tannic acids of wine The bacteria just described are, however, doubtless to be considered as the chief cause, though a direct inoculation of them does not appear to produce the disease

According to Voisenet, there exist in water several species of bacteria which appear to bring about the bitterness of wine. He showed that his water bacteria and the bacteria detected in bitter wines possess one property in common, that of converting glycerine into acrylic aldehyde, a substance which he found present in all wines affected with bitterness properly so called One of these species is called by him *Bacillus amaracrylus*, another is characterised as follows —More or less motile, able to develop at a temperature of 42° C in broth containing 0·1 per cent of carbolic acid, coagulates milk, evolves hydrogen and carbonic anhydride in a lactose broth Colonies on gelatine excessively small, do not liquefy the gelatine

Finally may be mentioned the **butyric acid taint,** with which very abnormal wines are sometimes affected.

We next come to the **decomposition of acid,** as effected in wine by certain bacteria and, to a less extent, by yeasts This interesting phenomenon has been thoroughly studied of late years and was found to be in some cases a consequence of certain diseases, such as the lactic acid taint, which begins with a decrease in the amount of acid, the bacteria afterwards attacking the sugar and forming lactic acid and acetic acid The decomposition, however, occurs more frequently in perfectly sound—and especially full-bodied—wines, *with a favourable effect on the quality.* A. Koch was the first to establish that the phenomenon is caused by bacteria (1910),

shortly afterwards it was proved by Kunz and Moslinger that normal wines may contain as much as 4 to 6 per cent of lactic acid, and that in proportion as this acid increases, the malic acid disappears. Later, from a perfectly sound wine with decreasing acidity, Seibert isolated a micrococcus (*M. malolacticus*) which was found to decompose malic acid and to form lactic acid and a small quantity of volatile acids.

Very extensive and thoroughgoing investigations into this important question were carried out by Muller-Thurgau and Osterwalder, both on fruit and grape wines. One result was to prove that several bacterial species are capable of decomposing and fermenting the malic acid of wine, with formation of lactic acid and carbon dioxide, the latter producing a froth, which, especially at high temperatures, may give the appearance of alcoholic fermentation; the phenomenon has, therefore, often been regarded as a kind of "secondary fermentation." The milder taste acquired by the wine may be accounted for by the fact that both by titration and on the palate the lactic acid formed has only half the acidity of the malic decomposed. They further made the interesting observation that a decomposition of acid could be brought about by the addition of a pure culture of one of these bacteria (*B. gracile*) to sterilised fermented wines.

In Swiss wines of comparatively high acidity, Muller-Thurgau and Osterwalder observed that the decomposition of acid was accelerated at certain temperatures. Thus, at 15° C. it set in so early and went on so quickly, that these wines only required keeping for a few weeks at this temperature. It is, however, between 20° and 26° C. that the decomposition begins and progresses most quickly in other wines; temperatures below 10° C. have proved to be unfavourable.

The bacteria tending to decompose acid are checked in their growth and action by the acids occurring in the wine. The more the grape juice has been freed from acid by the action of calcium carbonate, the sooner the decomposition will set in; the acid is, therefore, first partially neutralised, and then the desired decomposition brought about by the action of micro-organisms.

B. gracile is the most active and most frequently occurring species concerned with the decomposition. It is a very frail and tender organism; its habitat is on grapes and other fruits, where it is found freely.

In Swiss red wines the authors of this work record some peculiar morbid changes after completion of the decomposition of malic acid. The wine acquires a flat or dull, sometimes a bitter, taste, becoming at the same time discoloured and poor in extract. This disease is caused by a peculiar species, *B. tartarophthorum*, which attacks tartaric acid and also glycerine; the tartaric acid disappears completely; acetic acid and propionic acid are formed.

The disease can be provoked by addition of pure cultures. In some cases glycerine only is decomposed.

The bacteria concerned are classified by Muller-Thurgau and Osterwalder on the following lines —

I. Rod and Filament Bacteria.

Mannite bacteria, ferment lævulose, with formation of mannite, lactic acid, acetic acid, and carbonic acid

A 0 7 to 1 3 μ thick Rapid decomposition of xylose

(*a*) Decomposition of 1-arabinose, citric acid, not lactose

B mannitopœum, Muller-Thurgau On gelatine it forms colonies with irregularly lobate edges and fibrous structure on the surface, in gelatine, smooth edges Ferments lævulose, dextrose, and galactose, with formation of lactic, acetic, and carbonic acid Slow decomposition of malic acid Does not liquefy gelatine Optimum temperature between 26° and 34° C Facultative anaerobic

(*b*) Decomposes lactose, but not 1-arabinose or citric acid

(*α*) Decomposes dextrose, with formation of acetic acid, does not attack malic acid.

B Gayoni, Muller-Thurgau and Osterwalder (*ferment mannitique Gayon*) Isolated by Gayon and Dubourg from an Algerian wine Colonies frail, with fringed edges. Does not liquefy gelatine Ferments lævulose, dextrose, and galactose, with formation of lactic, acetic, and carbonic acid, ferments also saccharose, maltose, lactose, and raffinose Facultative anaerobic

(*β*) Decomposes dextrose, with formation of small quantities of acetic acid Vigorous decomposition of malic acid with formation of lactic and carbonic acid

B intermedium, Muller-Thurgau and Osterwalder On yeast deposit it gives rise to flocculent formations consisting of long threads, in the deposit are seen zoogloea-like aggregates, surface colonies snow-white, edges fringed and lobate, non-liquefying. Ferments lævulose and xylose, with formation of lactic, acetic, and carbonic acid, in the case of lævulose followed by formation of mannite Decomposes dextrose, galactose, saccharose, lactose, maltose, raffinose, with formation of lactic and carbonic acid, a little acetic acid and alcohol Facultative anaerobic

B 0 4 to 0 6 μ thick Does not attack xylose, actively decomposes malic acid, with formation of lactic and carbonic acid

B gracile, Muller-Thurgau and Osterwalder The most frequent of the acid-decomposing species A frail organism, which, though very active, forms but a small deposit a snow-white, slimy coating, which when the bottle is inclined will slide on the rest of the deposit Short rods, and also threads, often curved Zoogloea

On gelatine, colonies with smooth edges, non-liquefying Ferments lævulose, dextrose, and galactose, with formation of lactic, acetic, and carbonic acid, with lævulose it forms mannite, with dextrose and galactose ethyl alcohol Optimum 22°-26° C. Facultative anaerobic

A peculiar position is occupied by *B tartarophthorum* Rods and threads 0 8 to 1 μ thick Decomposes tartaric acid Partially decomposes glycerine Converts tartaric acid into acetic acid, a little propionic acid from glycerine

II. Cocci, Diplococci, and Tetrads.

Decomposes malic acid into lactic and carbonic acid

A Ferment dextrose and lævulose, with formation of lactic acid and a little acetic acid

(*a*) 0 5 to 0 7 μ thick Attacks maltose and lactose, but not amygdaline

M acidovorax, Muller-Thurgau and Osterwalder Single cocci, diplococci, and tetrads, zoogloea Surface colonies on gelatine roundish, smooth edges Decomposes dextrose, lævulose, galactose, lactose, and maltose, with formation of lactic acid Optimum 26 5° C Facultative anaerobic

(*b*) 0 7 to 1 5 μ thick Attacks amygdaline very actively, but not maltose or lactose

M variococcus, Muller-Thurgau and Osterwalder Single cocci, diplococci, and tetrads, zoogloea Surface colonies smooth edges Decomposes lævulose, dextrose, and galactose, with formation of lactic acid Optimum 26 5° C Facultative anaerobic

B Ferments dextrose, with formation of volatile acid, not lactic, does not attack lævulose.

M malolacticus, Seifert 1 0 μ thick
Cocci of same size as those of *M gracilicoccus* Identical action on malic, tartaric, citric, lactic, and succinic acid, but differs as described in its action on hexoses
(*M saprogenes vini* and *Bacillus s v*, isolated by Kramer, are not included by these authorities among the wine bacteria proper, because they are found in strongly decomposed wines in a putrid state)

In **leaven,** lactic acid bacteria also occur which, without doubt, play a part in the fermentation of bread Peters, for instance, found a species which occurs in motile rods, and forms a slimy skin on neutral yeast-water-sugar at 30° C Henneberg found Leichmann's *Bact lactis acidi* by development in mash at 48° C, and at 38° C a special species which he named *Bact panis fermentati*, which occurs in mash in short and long rods, and forms small white colonies on wort-agar. The optimum for the production of acid is at first about 37°-42° C., and afterwards 34°-38° C.

The lactic acid fermentation plays a very important part in the means adopted in different countries for **preserving vegetable foods** for both man and beast. Vegetables are chopped up, in certain cases salt is added, and they are placed in vessels or in hollows protected from access of air A fermentation sets in and lactic acid is produced as one of the products. This acid protects the material from the attack of other micro-organisms, and gives the peculiar character to the preserved vegetables The temperature usually rises when fermentation begins, which allows of the partial development of special thermophilous bacteria As a consequence of the development of other micro-organisms, the amount of acid is always reduced with prolonged fermentation. The active species are described by Wehmer, Aderhold, Weiss, Henneberg, and others A rich flora of species occurs, however, in such ferments

On these different media Fred and Peterson found a particularly interesting organism, *Lactobacillus pentoaceticus*, which is closely allied to the mannite bacteria described by Gayon and Dubourg, and also by Müller-Thurgau It takes the form of rods with blunt ends, 2 to 3 times as long as they are broad, of varying size, it also occurs as filaments, apparently feebly motile, not spore-forming On agar-plates it forms small colonies resembling common lactic acid bacteria. It is characteristic of this species that it very actively ferments *xylose*,* with full or limited access of air The main products are lactic and acetic acid, optimum temperature about 27° C. When fermenting glucose, galactose and mannose, it chiefly forms lactic acid and ethyl alcohol, with evolution of carbonic acid In the early stages of its fermentation of fructose, it forms considerable quantities of mannitol, lactic and acetic acid, the mannitol gradually disappears, and fresh quantities of lactic acid are formed The species can be developed by introducing a sample

* It will be remembered that lactic acid bacteria containing this ferment were previously discovered by Kayser

of green corn ensilage, manure, sauerkraut, etc, into a yeast decoction to which xylose and an excess of calcium carbonate have been added The species seems to be widely distributed, and plays an important part in the production of corn ensilage (green corn fodder), its action being far more powerful than that of *Bac acidi lactici* and *B Bulgaricum.* Crolbois found an apparently similar species, which proved to be particularly active in preserving green fodder

Kephir, on which the investigations of Kern have thrown some light, is an effervescent, alcoholic sour milk, prepared by the inhabitants of the Caucasus from cow's, goat's, or sheep's milk. It is made by adding a peculiar ferment, "kephir-grains," to milk These are white or yellowish and irregularly-shaped grains, not larger than a walnut and of a tough gelatinous consistency, and when dried become cartilaginous and brittle. The essential part of the grains consists of rod-like bacteria, connected in threads, and enveloped in gelatinous membranes. Kern calls this bacterium *Dispora Caucasica* According to Beijerinck this species, which he calls *Lactobacillus caucasicus,* produces in lactose, saccharose, glucose, and maltose a direct lactic acid fermentation It produces solid, nodular colonies on whey gelatine resembling the kephir-grains Besides bacteria, various yeast fungi and, frequently, moulds occur in the kephir-grains

In the preparation of kephir a little milk is first poured on the grains and allowed to stand for twenty-four hours at about 17° C, the milk is then poured off, and the grains preserved for future use This milk is mixed with fresh milk, and poured into closed bottles, or leather sacks, the fermentation is completed in two or three days if the liquid is frequently shaken. It now contains about 2 per cent of alcohol This result is probably brought about by the simultaneous action of *Dispora* and yeast cells in combination with lactic acid bacteria present in milk These ferments convert a portion of the lactose into lactic acid, the alcohol and a part of the carbon dioxide result from the action of yeast As the fermented milk, according to some authorities, contains less coagulated casein than ordinary sour milk, it may be assumed that the *Dispora* is also able to partly liquefy (peptonise) the coagulated casein, perhaps with the help of the gelatinous mass secreted by the bacterium, and found in the kephir-grains, but not present in the fermenting milk. According to investigations of Hammarsten, however, the amount of casein does not appear to decrease, but a part of it undergoes certain alterations, partly physical, in consequence of which it becomes more finely flocculent The want of agreement in these results may possibly originate in the different biological composition of the selected kephir-grains.

Freudenreich regularly found in a number of kephir samples *Dispora Caucasica* (*Bact. Caucasicum*), which readily developed on

milk-agar plates and in lactose broth at 35° C, the bacilli frequently have glistening points at both ends, and Freudenreich assumes that this phenomenon coincides with what Kern regarded as spores; unmistakable spores, however, were never found.

Two lactic acid coccus forms and a yeast species also occur in all samples. One of the cocci (*Streptococcus a*) forms diplococci and chains, and produces in lactose gelatine large, white colonies, with coarse granulation at the edge; the best temperature for the growth of this species is about 22° C.; it coagulates milk most rapidly at 35° C, and contributes essentially to the production of a sourish taste and fine flocculent appearance. The other coccus (*Streptococcus b*), likewise forming diplococci and chains, occurs in smaller colonies than *a*, and, in contrast with the latter, grows well at higher temperatures, and forms more acid than *a*, but does not coagulate milk. If this species is transferred, together with the kephir-yeast, to lactose broth, the fermentation is more vigorous than if the bacteria alone are inoculated; Freudenreich, therefore, presumes that *Streptococcus b* splits up lactose, and that its fermentation is rendered possible by the kephir-yeast. The *kephir-yeast* (a *Torula*) discovered by him grows remarkably well and gives a weak fermentation in beer-wort, but does not appear to produce any fermentation in milk or lactose broth. The growth consists of oval cells (3 to 5 μ long, 2 to 3 μ broad); it forms neither film nor spores, and its optimum temperature lies at 22° C.

In the course of his experiments, Freudenreich succeeded in producing a liquor resembling kephir, for which purpose he inoculated a mixture of the four species in milk, and, after a lapse of some days, introduced a small portion of this sour, coagulated milk, which had been repeatedly shaken, into sterilised milk; he therefore concludes that these four species, through their symbiosis, are able to bring about the kephir-fermentation. He could not observe any synthesis of kephir-grains, and it is not yet clear what part *Dispora Caucasica* plays in the whole process; moreover, it appears to be highly probable that species of bacteria, other than the two coccus forms described by Freudenreich, in addition to other budding fungi, are active in the process. It may be deserving of notice that in the author's laboratory it has been proved that a genuine *Saccharomyces* (*S. fragilis*) occurs in Russian kephir-grains which ferments milk-sugar directly, whereas all previous investigators only found budding fungi incapable of spore-formation.

Kuntze isolated from kephir-grains a large number of species, all of which he considers to be necessary for the formation of the grains. Thus, besides common lactic acid streptococci he found some bacteria belonging to the aerogenes group, further a number of yeast species (including several which do not ferment lactose), the action of which he believes to consist in regulating the course of fermentation and furthering the growth of lactic ferments;

finally, various butyric acid bacteria, some of which dissolve casein, while others tend to form slime. By mixing up all the species, Kuntze succeeded in preparing kephir-grains which on addition to milk converted it into the well-known beverage.

Koumiss is a similar fermented milk, prepared chiefly from mare's milk by the nomadic tribes of Southern Russia and Siberia, it has been applied in many countries as a cure for various diseases. The true Koumiss, as prepared by the nomads, is fermented in leathern bottles, fermentation being started by adding a little dried milk from a previous fermentation The organisms present sour and coagulate the milk during their development, and an alcoholic fermentation sets in, with evolution of gas. The coagulated mass is so finely divided that the liquid only turns thick An accurate examination of the active organisms was undertaken by Schipin, who proved the constant presence of a yeast species, a lactic acid bacterium, and a special species of bacteria which occurred in large quantities, and appears to be characteristic of the Koumiss fermentation It is a facultative anaerobe which forms whitish colonies in gelatine, consisting of a central nucleus with streamers in all directions. It thrives best on sour-milk gelatine, and does not liquefy the gelatine By the addition of cow's milk at 37° C. it coagulates to a thick paste without noticeable separation of whey. Its optimum lies between 20° and 30° C. Ten minutes' heating at 60° C is sufficient to kill it In experiments with mare's milk in presence of these three organisms, Schipin arrived at the conclusion that this species plays the most important part in the formation of Koumiss, and that it produces a lactic acid, as well as an alcoholic fermentation and peptonises the albumen It only displays its activity when the yeast and lactic acid bacteria have prepared the way for its development

It appears that the organisms occurring in Koumiss samples of different origin are far from being identical Thus, Rubinsky, continuing his researches, found two species, living symbiotically, to be prevalent and particularly active—viz, first, a yeast which ferments lactose and produces about 0 4 per cent. of lactic acid in milk, it converts casein and albumen into albumoses and peptones, and produces aromatic, ester-like substances: secondly, a "Koumiss bacterium," belonging to the third group in Lohnis' system, and exhibiting very different forms, from streptococcus-like cells to very long filaments, it has little power of acid production, temperature limits 23° and 40° The metabolic products of the yeast considerably further the growth and acid production of the bacterium. By infecting horse's or camel's milk with pure cultures of these two species, Rubinsky could prepare a normal Koumiss, but not from cow's milk. Finally, he found such widely occurring streptococci as *B. acidi lactici Hueppe* and *B. Caucasicum*,

but only in comparatively small quantity and without any direct influence on the true fermentation

Armenian **Mazun,** a fermented milk (of buffaloes, cows, goats) resembling kephir, is drunk, and also employed for making butter and cheese. Emmerling and Kalanthar, and later Duggeli, discovered in it various bacteria, moulds and yeasts, the latter being closely studied by the two latter, who found a temperature of 30° C. to be the most favourable for the manufacture and propagation of mazun Among the three yeast species observed by Düggeli, one proved to be of particular importance It exhibits oval and elongated forms, and on whey-agar forms white colonies with a dull, almost mealy surface, if grown in milk, it produces slight amounts of acid and alcohol, and at the same time an agreeable odour of sebacic acid esters Mazun was further examined by Weigmann, Gruber, and Huss, who also detected a Pastorianus yeast, fermenting lactose, together with two particularly characteristic bacteria —*Bacillus Mazun*, spore-forming rods with proper motion, 2 to 4 times as long as they are broad, spores oval-oblong. It will grow under aerobic as well as anaërobic conditions, on gelatine it forms grayish-white, liquefying colonies with off-shoots radiating in all directions, on agar grayish colonies with lobate edge. Coagulates milk *Bacterium Mazun* forms long rods without proper motion, both aerobic and anaerobic It grew only on whey-agar plates (20° to 37° C), where it formed small white colonies, round or irregular, of a greasy lustre Produces abundant acid, and is considered to be specifically characteristic of mazun, in conjunction with the first-named, strongly peptonising species, which imparts a cheese-like flavour to the milk

In a similar way in Egypt, a sourish aromatic product resembling kephir is prepared from buffalo's, goat's, and cow's milk named **Leben.** It contains less alcohol than kephir, and coagulates As in previous cases, boiled milk is brought into fermentation by the addition of dried milk from a previous fermentation. According to Rist and Khoury five different species are active in this fermentation, a *Streptobacillus* which coagulates milk and produces lactic acid from lactose. a very thin *Bacillus* which also yields lactic acid, a *Diplococcus* which strongly coagulates milk; a yeast species which ferments glucose, saccharose, and maltose, but not lactose, but which, along with the *Streptococcus*, may give a vigorous fermentation in milk, as the bacterium hydrolyses lactose; and, finally, a *Mycoderma* species which can ferment glucose and maltose, but not lactose The *Streptococcus* and *Diplococcus* also possess a special rennet enzyme According to Rist and Khoury, by the use of these five species, Leben can be prepared from milk, and, best of all, if the two budding fungi are added first and the bacteria later

Yoghourt is a species of sour milk or thick milk prepared in

Turkey and Bulgaria. Sheep's or cow's milk is used, which is boiled and reduced by evaporation to half its volume, then cooled to 45° C, and the ferment—"Maya" or "Podkvassa"—is added. This consists of milk residues from previous preparations, dried under special conditions and ground, and contains many species of bacteria After a fermentation lasting for nine to sixteen hours at a temperature of 40° C, the Yoghourt is ready for consumption It is more or less solid, according to the degree of concentration, and possesses a sourish aromatic taste. It is eaten cold, either alone or with the addition of rice, bread, sugar, or fruit syrup. We owe the first bacteriological investigation to Grigoroff, who found three different lactic acid bacteria The most important is *Bacillus A* (*Bact Bulgaricum*) It forms long motionless rods, often linked in chains, grows well on saccharine substrata, has an optimum temperature of 45° C It produces alcohol, and attacks lactose, mannite, dextrose, maltose, and lævulose, but not rhamnose, dulcite, and sorbite *Micrococcus B* occurs as single cocci or diplococci. In addition to the above varieties of sugar, it attacks rhamnose and glycerine *Streptobact C* forms short rods linked in chains It attacks lactose, saccharose, lævulose, and glycerine, but not maltose, mannite, rhamnose, dulcite, or sorbite The optimum temperature for the last two species is 45° C., and they produce alcohol

After Grigoroff, several workers examined the Yoghourt bacteria, more particularly that species known as *B Bulgaricum,* a name which has proved to include a number of strains differing slightly from each other This species surpasses all other known lactic acid bacteria in its power of producing lactic acid, the quantity produced amounting sometimes to 30 g per litre Bertrand and Weiswiller showed that it forms small quantities of succinic, acetic, and formic acid Metschnikoff, having established that it can be acclimatised in the human intestine, and that it is innocuous to the system, suggested that a pure culture of this Yoghourt bacterium, grown in milk, should be introduced into the intestine, in order that the large quantity of lactic acid formed might check the growth of the numerous toxic bacteria, which develop more particularly in the lower intestine The results arrived at by Grigoroff and Metschnikoff were confirmed by Cohendy, who showed that the species will coagulate milk in 18 hours at 37° C. Klotz observed that at its optimum, 45° C, it is able to coagulate milk in five hours Kuhn described a "Granular Bacillus"* which, however, Kunze is inclined to regard as a mere variety According to Severin, the species is identical with *Streptobacillus lebenis,* isolated from Egyptian Leben, he also discovered some slime-forming varieties.

* The granules, according to Oehler, on coloration with alkaline methylene-blue, acquire a red colour, whereas the filaments are coloured blue They also become conspicuous by Neisser's stain

The existence of differences between the strains examined is also shown in their reaction on the sugars, some of them fermenting saccharose and maltose, while others do not Barthel found the products of three strains derived from Vienna, Berlin, and Paris, differed in rotatory power two of them produced lævo-rotatory, the third dextro-rotatory acid L hnis proposes to give all those long-rod lactic acid bacteria which are isolated from fermented milk the common denomination *B Caucasicum*, after the form first isolated by Beijerinck from kephir, all the strains being thus regarded as a single species Some workers have also detected yeast species in Yoghourt, and attach more or less importance to them

3. Butyric Acid Bacteria.

When stale milk in which lactic acid bacteria have developed is neutralised by the addition of calcium carbonate, so that calcium lactate is formed, it will, as a rule, undergo a buytric fermentation. Pasteur showed in 1861 that this fermentation is brought about by particular micro-organisms which are able to live without air ("*vibrions butyriques*") This spontaneous butyric acid fermentation takes place most vigorously at 35°-40° C Starch, glycerine, dextrin, cane-sugar, maltose, lactose, and dextrose are likewise decomposed by the butyric acid ferments, and such fermentations are of frequent occurrence, as the bacteria belonging to this group are very widely distributed in nature To induce a butyric acid fermentation, Fitz recommended using a mixture of 2 litres of water, 100 grammes of potato-starch or dextrin, 1 gramme of ammonium chloride, the ordinary nutrient salts, and 50 grammes of chalk, this mixture is to be maintained at 40° C Bourquelot recommends exposing slices of raw potatoes, standing in water for two or three days at a temperature of 25°-30° C.

Beijerinck proceeds as follows:—5 per cent of finely ground fibrin is added to a 5 per cent solution of grape-sugar After vigorous boiling, it is inoculated with garden soil, and immediately placed in an incubator at 35° C The fermentation will set in within a day or two The liquor is then neutralised with soda solution A growth is thus obtained of Beijerinck's *Granulobacter saccharobutyricum*, the majority of other bacteria being destroyed by boiling, or else checked by the butyric acid fermentation. For Bredemann's method of cultivation and regeneration, see the end of this chapter

The most important products of the butyric acid fermentation are butyric acid, carbon dioxide, and hydrogen

According to Pasteur's experiments, the butyric acid ferment can perform its functions without access to free atmospheric oxygen. The usual spontaneous butyric acid fermentations proceed most

vigorously when oxygen is excluded. It has, however, been shown by recent experiments that there are many butyric acid bacteria which multiply and induce butyric acid fermentation when they have access to oxygen—aerobic species. In the course of years a very large number of butyric acid bacteria have been described. By the study of this mass of material, it has been shown that they are divisible into two groups—first, the **true butyric acid bacteria**, being those that produce butyric acid as the chief product of fermentation by decomposition more especially of carbohydrates or

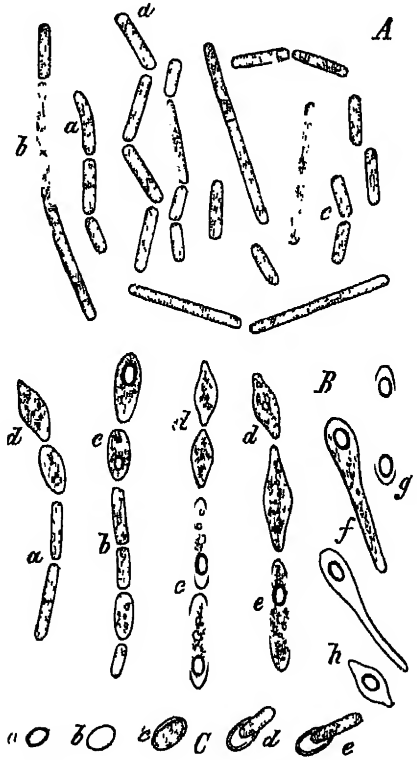


Fig. 24.—*Clostridium butyricum*, Prazm. (after Prazmowski).—*A*, Vegetative state; *c*, short rods; *d*, long rods; at *a* and *b*, rods and filaments curved like vibriones. *B*, Formation of "resting spores": *b*, *d*, rods, previous to; *c*, *e*, during; *f*, *g*, *h*, after the formation of resting spores; *c*, elliptical; *d* and *h*, lemon-shaped; *e*, *g*, spindle-shaped; *f*, tadpole-form; at *a* rods still in their vegetative state. *C*, Germination of resting spores; the spore *a* expands into *b*; *c*, shows the differentiation of the membrane into exo- and endosporium. The contents surrounded by the endosporium issue from the polar fissure of the spore in the form of a short rod (*d*), which appears prolonged at *e*.

calcium lactate; and, secondly, there remain many species which form butyric acid along with other products by the breaking down of albuminoids. This applies particularly to putrefactive bacteria,

many of which only produce minute quantities of butyric acid. Thorough chemical investigations have been carried out by Fitz, and more recently by Perdrix, as well as by Schattenfroh, Grassberger, Bredemann, and Kirnow, who investigated the action of a number of species upon starch, the sugars, glycerine, cellulose, and the albuminoids, and determined the products of fermentation.

One of the first species to be minutely described is Prazmowski's *Clostridium butyricum* (*Bac. butyricus*, Fig. 24). It occurs in the form of short and long threads and rods, which may be either straight or somewhat curved. The rods are in brisk movement, and under a strong magnifying power they are seen to be covered with a large number of cilia (Fig. 25). Before the formation of spores in the rods, the latter swell and form peculiar spindle and lemon-shaped, elliptical, or club-like forms, as shown in the diagram; at the same time they are coloured blue by iodine. The spores can withstand boiling for five minutes. On germination the spores burst their outer envelope, and the germ filament grows in the same direction as the longitudinal axis of the spore. *Clostridium butyricum* grows most vigorously at a temperature of about 40° C., and may then rapidly become predominant in sugar solutions if the lactic acid ferment has previously converted a portion of the sugar into lactic acid. This species is decidedly anaerobic.

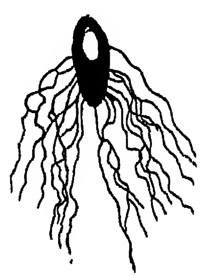


Fig. 25.—*Clostridium butyricum* (after A. Fischer), with endospore and stained cilia.

Fitz has described a species belonging to the aerobic organisms a bacillus of a short cylindrical form, which is not coloured blue by iodine, and is motile in a moderate degree. It ferments all carbohydrates with the exception of starch and cellulose.

According to Fitz the spores of butyric acid bacteria can withstand the temperature of boiling water for a length of time, naturally dependent, as in all cases, on their condition and on the nature of the substratum; Fitz gives three to twenty minutes as limits. They can, however, be killed at a lower temperature if maintained long enough; thus they are killed by being heated for six hours at 90° C. in a solution of grape-sugar; but in glycerine, at the same temperature, for a period varying from six to eleven hours.

Hueppe has likewise described a species (*Bac. butyricus*) found in milk, and occurring in the same forms as the species discovered by Prazmowski, but it proved much less sensitive to oxygen. This species does not, however, form butyric acid from carbohydrates, but from albuminoids.

Another aerobic species, *Bac. boocopricus*, was detected by Emmerling in cow dung. It forms short rods, and is characterised by not liquefying gelatine, and by converting glycerine into butyric acid.

Gruber found three well-defined species associated under the name of *Clostridium butyricum*, two of which are exclusively anaerobic One of the latter species consists of straight or slightly-curved rods, which become spindle- or barrel-shaped during the formation of spores In nutrient gelatine it forms colonies which, when seen in reflected light, appear brownish-black or black The second species consists of strongly-curved rods, at the end of which spores appear, it forms yellowish or yellowish-brown colonies The third species is also capable of growth and of causing fermentation in the absence of oxygen, its development is, however, decidedly assisted by the presence of oxygen, and it is only then able to produce spores The rods are cylindrical, with the formation of spores they become spindle-shaped, and in the centre of the spindle the large spore is formed The colonies in nutrient gelatine are yellowish All three species form butyric acid and butyl alcohol from carbohydrates

In the water supply of Paris, Perdrix found an anaerobic bacterium (*Bacille amylozyme*), which occurs in the form of motile threads, four to six times as long as they are broad This converts saccharose into acetic and butyric acids, with evolution of hydrogen and carbon dioxide, and it also produces amyl and ethyl alcohols. The optimum for its growth is 35° C On slices of potato it forms whitish round colonies, which gradually liquefy the substratum. This species is very sensitive to acids The spores can withstand ten minutes' heating to 80° C

Bac orthobutylicus was isolated by Grimbert from seeds of the leguminosæ It was separated by heating for one minute at 100° C Its spores survive this treatment The species is anaerobic, and forms motile rods two to four times as long as they are broad, with rounded ends In these rods two and three spores may occur Its fermentation products are chiefly butyric acid, butyl alcohol, and acetic acid, together with carbon dioxide and hydrogen It ferments saccharose, maltose, lactose, and glycerine It hydrolyses starch, and converts dextrine into maltose Grimbert proved by detailed experiment that the length of fermentation, the concentration, the reaction of the liquid, and the conditions of growth all influence the amounts of the fermentation products Thus with an acid reaction the amount of alcohol increased and the formation of acid simultaneously decreased On the other hand, the quantity of alcohol diminished and that of acid increased when the liquid was neutralised with calcium carbonate

Amongst other workers in this field may be mentioned Botkin, Flugge, who isolated a species from milk by heating for one and a-half hours in boiling water or in a current of steam, the spores surviving this treatment, v Klecki, who cultivated *Bac. saccharobutyricus* from cheese

Clostridium Pasteurianum, discovered by Winogradsky, is of

particular interest. He isolated it from garden soil by heating for ten minutes at 75° C., and then cultivating in a stream of nitrogen in a substratum free from nitrogen. The species can, therefore, *absorb free nitrogen from the air* and assimilate it. It forms butyric acid, acetic acid, minute quantities of alcohol, carbon dioxide, and hydrogen, and occurs as short, thick motile rods, which at a later stage expand into spindle shapes, and during the production of spores gives a violet-brown colour with iodine. The free spores are surrounded by an irregular mass of jelly.

Beijerinck has drawn special attention to the possibilities of butyric acid bacteria which display bodies resembling granulose in the swollen cells and may be coloured with iodine, and has formulated a group which he calls *Granulobacter*. The originator of the butyric acid fermentation, the preparation of which has been described, he calls *Granulobacter saccharobutyricum*; it forms varying quantities of butyl alcohol, carbon dioxide, and hydrogen from saccharose, better from glucose, and also from maltose, and it secretes diastase.

Schattenfroh and Grassberger examined a long series of species, both pathogenic and non-pathogenic, and found that the latter consisted chiefly of two species, one of which is motionless, and is very widely distributed. It forms both short and long rods, particularly on alkaline substrata containing starch. It exhibits the granulose reaction in the *Clostridium* form (this usually disappears with the formation of spores), and it liquefies gelatine. The other species is motile, and forms thin rods with from six to twenty cilia on each. They are also motile in the spore stage, and do not liquefy gelatine. Neither of them attacks cellulose. We must here recall *Paraplectrum foetidum* (Weigmann), which is widely distributed in milk. It coagulates the milk, and then dissolves the coagulated mass, and develops a very objectionable smell of cheese.

By extensive researches on original cultures supplied by fellow-workers, and also on material sent from all parts of the world, Bredemann, with a full knowledge of the literature, came to the conclusion that a large number of the *many species described are identical*. For the numerous strains in question, he proposed the specific name suggested earlier by Van Tieghem: *Bacillus amylobacter*. They all possess the power of *combining with atmospheric nitrogen*. Winogradsky has shown, however, that they are very apt to lose this power by artificial cultivation. The description of this bacterial type by Prazmowski (see above) may be accepted: Short and long motile peritrichous rods of varying thickness. They sometimes appear able to divide themselves so as to evolve short roundish cells resembling cocci. The form of the spore is most frequently cylindrical, more rarely bean-shaped, oval or round; the spore is usually surrounded by residual sporangium membrane. Length about 2 μ, breadth 1 μ. Germination polar.

In the course of spore-formation the rod assumes spindle shape or other forms, sometimes very irregular The spore is often, but not always, found near one of the poles, which is swollen The young thin rods are coloured uniformly yellow by iodine, which, as already shown, can produce in the contents of the thicker rods the same reaction as starch, hence the name *amylobacter* They also give the glycogen reaction These bodies, which are distributed in the form of granules, generally, or at least partially disappear in the course of the development of the spore On coloration with methylene blue (one volume of a saturated solution in 95 per cent alcohol to 9 volumes of water), old cells especially often exhibit formations that may be interpreted as cell-nuclei Optimum temperature of examined strains 30° C, both for the germination of the spore and the growth of the organism The spore will germinate even at a concentration of about 20 mg of oxygen per litre

For the development of these bacilli Bredemann recommends putting 2 g of earth in a test-tube filled to a height of 6 cm. with Winogradsky's non-nitrogenous solution * Heat to 80° C. for ten minutes and allow to stand at 28° C. A violent fermentation will soon set in, and the tube is allowed to stand for eight days in an oblique position By that time the bacteria will have formed a film on the lower side of the tube A portion of this culture is diluted with water, the temperature raised to 80° C, and the dilution transferred to agar plates in a vacuum (1 mg of oxygen per litre) at 28° C (1 per cent of dextrose, 1·2 per cent. of Wittes peptone, 0·8 per cent Liebig's meat-extract, 0·2 per cent NaCl, 1·6 per cent agar, slightly alkaline) After spore-formation and before any fresh inoculation the temperature is raised to 80° C. for five minutes

As these bacteria are not strictly anaerobic, they will support up to 30 mg of oxygen per litre—they can be grown, not only under ordinary anaerobic conditions, but also in open flasks, where, if abundantly inoculated, they exhibit the same power of fermenting and fixing free nitrogen †

As regards the *fermentation products*, it is evident from Bredemann's researches that the differences established by many of the species described are largely attributable to different treatment and condition of the cultures Besides CO_2 and H, they produce butyric acid and several other volatile acids, lactic acid and various alcohols were found in varying proportions A characteristic feature which they have in common is, that they attack albumen and

* In one litre of water, free from ammonia 2 per cent dextrose, 1 g potassium phosphate, 0 2 g magnesia phosphate, infinitesimal doses of sodium chloride, ferrous and manganese sulphates, together with excess of chalk

† The following has proved to be a suitable nutrient liquid —1 0 g of di-potassium phosphate, 0 2 g magnesium sulphate, 0 2 g sodium chloride, 0 01 g ferrous sulphate, 0 01 g manganese sulphate, 20 g dextrose, 10 g precipitated calcium carbonate in 1,000 c c drinking water.

peptone without producing ill-smelling substances in contrast with the typical putrefactive bacteria.

As already stated, they are apt to lose their power of combining with atmospheric nitrogen. After living some time under the artificial conditions described, it is often difficult to obtain a normal development with fermentation and nitrogen fixation in a liquid free from nitrogen. In this respect the cultures behave differently. Just as is known to be the case with the Azotobacter living in the tubers of Leguminosæ, so it has proved possible to restore this power by letting the culture pass through earth. Dried and filtered garden soil is put into test-tubes, moistened with water and sterilised at 150° C. for three quarters of an hour; the soil is then freely inoculated from an agar culture diluted in sterilised water, and the mixture left to stand for some weeks, *in vacuo* or with access of air. A culture so treated will react like a fresh one isolated from earth, in a nutrient liquid free from nitrogen. The same result can often be obtained by adding a few grains of sterile earth to the usual non-nitrogenous liquid. An addition of small quantities of nitrogen compounds to the liquid does not prevent the bacteria from fixing nitrogen.*

There is no doubt that butyric acid fermentation may take place both in breweries, distilleries, and yeast factories, as well as in the fermentation of wine, which is probably caused by the activity of certain species of bacteria. Thus butyric acid has been detected in potato fusel oil and in cognac, as well as in the yeast mash of the distillery.

If the preparation of the mash and wort goes on under in different conditions, a good opportunity is afforded for the development of such bacteria, and this applies also to the higher temperatures at which top fermentation is carried on.

A bacterium that produces butyric acid together with other substances is *Bac. lupuliperda*, described by Behrens, which occurs

* Among the bacteria dealt with in this chapter belong those active in the *retting of flax, hemp, nettles, etc.* They attack the intercellular substance, so that the fibre is liberated. The action is regarded as a hydrolysis followed by fermentation of the pectose in the lamellæ between the cells (hence the specific name of two of the active species met with: *Plectridium* and *Granulobacter pectinovorum*). Pure cultures methodically prepared (in the laboratory of the author and elsewhere) and rationally applied on an industrial scale, have given good results; the fermentation, unencumbered with foreign micro organisms, could proceed successfully and promptly. A typical anaerobic species occurring in such fermentation (described by Carbone under the name of *Bac. felsineus*) forms on milk agar small, slender rods with oblong spores situated at one end of the rod; *Clostridium*-forms seldom occur. It will multiply readily at 37° C. on sterilised hemp with addition of yeast. The growth is further developed in potato decoction, to which it imparts an orange-yellow colour and an ester like odour. Such a culture is used to inoculate hemp which is being water retted at 37° C. Of recent years such fermentations have been carried out, particularly in Italy and France, with the use of pure cultures of *aerobic* bacteria by Rossi's method (*Bac. Comesii*, spore forming), with supply of air. Under these conditions fermentation is completed in a shorter time, and the risk of excessive retting is avoided. The anaerobic species adhering to flax and hemp were not checked by aeration; but the quantity of organic acids formed was found to be considerably smaller than it is when these bacteria are active.

frequently on hops The spontaneous heating of hops has been shown by Behrens to be due to the development of this and other organisms It consists of motile cocci and short bacilli which liquefy gelatine In nutrients free from saccharose it produces large quantities of ammonium compounds, and, in particular, trimethylamine (the smell of rotten herring) In presence of saccharose the nutrient solution soon turns sour, and butyric acid is formed. The species appears to have its chief habitat in the earth, and bears a close resemblance to *Bac fluorescens putidus*, designed by Flugge

4 Bacteria Fermenting Cellulose.

Bacteria fermenting cellulose are widely distributed There appear to be a large number of species or varieties * They constitute one of the principal agents in the rapid transformation of the enormous quantities of cellulose, existing on and in the soil, in vegetable residues, roots, etc , while pectins, pentosans, starch, and sugar are also attacked by these organisms Certain moulds have likewise been found to be more or less active in these reactions.

Thanks to the admirable researches of Omelianski, we have obtained a clear conception of what bacteria are responsible for this action He sowed horse dung and river mud on Swedish filter paper (pure cellulose), with the addition of 1 gram of chalk, 1 gram of potassium phosphate, 0 5 gram of magnesium sulphate, 1 gram of ammonium sulphate or phosphate, and a trace of sodium chloride to 1 litre of water The fermentation was carried on at 34°-35° C in flasks adapted for the cultivation of anaerobic bacteria After a time the filter paper was riddled with holes. He thus proved that two different fermentations of cellulose are set up, a hydrogen ferment and a methane ferment, and that these are produced by two different species of bacteria. Omelianski separated the two by heating the fermenting material for fifteen minutes to 75° C The hydrogen fermentation then proceeded, whilst before warming the methane fermentation took place The reason is that the spores of the methane bacteria develop more rapidly than those of the hydrogen bacteria If the liquid is heated to 75° C after the germination of the spores of the methane bacteria, the vegetative rods of these bacteria will be killed, and only the spores of the hydrogen bacteria will remain alive and germinate By repeated sub-culturing an approximately pure growth of one or other species may be obtained

The cause of the hydrogen fermentation is a thin bacillus, straight or slightly curved, which forms spherical spores at one swollen end. It is not coloured blue by iodine. The fermentation

* In the varied methods of culture about to be described different species doubtless occur

products consist of fatty acids, carbon dioxide, and hydrogen. The exciter of methane fermentation presents a similar microscopic appearance, but the threads are thinner and the spores smaller It is not coloured blue by iodine. Its fermentation products consist about half of fatty acids (butyric and acetic acids) and half of carbon dioxide and methane

Since the appearance of Omelianski's work, these bacteria have been exhaustively studied by many workers regarding their life-history as well as the methodical application of selected species to agriculture and perhaps even more to industry, as they are capable of converting cellulose into glucose. Among the more prominent workers in this line may be mentioned Groenewege, Hutchinson, van Iterson, Kellermann, Kroulik, Langwell and Hind, Löhnis, Macfadyen, Pringsheim and Scales.

These investigations prove that this group includes, among its most active members, not only markedly *anaërobic*, but also *facultatively anaërobic* and *aërobic* species, together with a special sub-group of **thermophilous** forms, energetically attacking cellulose at 60°-65° C

The *aerobic* species were detected by Iterson (1904), who set up such a fermentation by sprinkling in a Petri dish, between two layers of filter paper, pulverised $MgNH_4PO_4$, pouring upon the upper layer 100 c.c. of tap water + 0·5 g. K_2HPO_4, adding a little mud or humus, and leaving the dish to stand at 24–28° C. While the medium is being dissolved, yellowish brown stains are formed, in which appears a small motile, non-sporulating bacterium *Bact. ferrugineum*—and a micrococcus, which are believed to act symbiotically. According to Löhnis, pure growths of these bacteria can be obtained, by using a mixture of 100 c.c. tap water, 1·5 g. agar, 0 3 chemically pure cellulose, 0·1 $CaCO_3$, 0·2 $NaNO_3$, 0·1 $MgSO_4$, 0·1 NaCl, 0·1 K_2HPO_4 Development at about 37° C. The colonies of the cellulose-dissolving bacteria are surrounded by a translucent ring.

Lohnis and Lochhead made cumulative experiments with an aqueous solution of 0·2 per cent of basic slag, 0·02 per cent. K_2HPO_4, 0 01 per cent. $MgSO_4$, 0·001 per cent. NaCl, and a nitrogenous substance such as meat-extract, in which strips of paper were soaked. The liquid was slightly alkaline or neutralised by hydrochloric or lactic acid. The solutions were inoculated with garden soil or with cow dung, and kept at 37° C. Whenever the liquid was rendered turbid by bacterial growth it was renewed, and at last uniform growths were obtained, consisting mainly of thin rods and filaments. On cellulose agar they grew weakly, with a clear zone. When transferred to paper, they attacked it, and at the same time there was observed the formation of darkly stained spherical bodies, which might again seem to indicate an obligatory symbiotic relation—if indeed these spherical bodies are not a

stage in the development of the thin rods, as would appear to be the case from Hutchinson and Clayton's researches (*Journ Agric. Science*, 9, 1919)

Extensive work was done by Groenewege (in Java) on some aerobic species from a cess-pool, which were grown by inoculating drinking water containing KNO_3 and K_2HPO_4 (0 25) and filter paper at 37° C After growing them subsequently on broth-agar at the temperature of the room, he observed a number of rods, both spore-forming and asporogenous They secrete an enzyme—cellulase—which will hydrolyse cellulose and convert it into cellobiose, this disaccharide is attacked by a second enzyme—cellobiase—and transformed into glucose In neutral or slightly acid liquid, acetic, butyric, and lactic acid are formed In alkaline liquids the sporogenous species will grow very freely, rapidly transforming cellulose into cellobiose, with formation of formic, acetic, and probably valerianic acid

During the aerobic decomposition of cellulose in the soil, Groenewege observed a symbiosis between the true cellulose bacteria and those which denitrify their decomposition products In this co-operation the cellulose disappears much sooner than when the cellulose bacteria act alone, a fact which illustrates the great importance of symbiosis in nature

Several aerobic species were isolated by Kellermann, Scales, and others by cultivation on cellulose-agar The cellulose was prepared by putting 5 g of filter paper into 100 c c of concentrated sulphuric acid diluted with 60 c c distilled water, after dissolving the cellulose by shaking vigorously, the liquid is quickly diluted to 2 litres with cold tap water, the cellulose is precipitated, and is filtered and washed The bacteria isolated were rod-shaped, and mostly provided with cilia Though fermenting cellulose most quickly under aerobic, they were also active under anaerobic conditions, and formed no gaseous products

These aerobic bacteria have been applied in agriculture Hutchinson, for instance, isolated a *Spirochaete* form which grows freely when abundantly supplied with air and preferably with inorganic nitrogenous matters (0 7 per cent ammonium sulphate), optimum temperature 30° C Straw thus treated, after being converted into friable compounds—without, however, undergoing a full decomposition—was tested on a large scale and found to compare favourably with natural farmyard manure

A special group of **thermophilous** species have their optimum at 60°-65° C The first observations are due to Macfadyen and Blaxall (1899), who sowed soil in nutritive liquids and, after a development of bacteria had set in, transferred the liquids to pure cellulose, filter paper and esparto cellulose The most active development and decomposition took place under anaërobic conditions at 60° C. Acetic and butyric acid were formed More recent work

by Pringsheim has shown that these thermophilous species attack cellulose very energetically, the decomposition products being cellobiose and, ultimately, glucose. He pointed out the formation, in varying proportions, of acetic and formic acid, together with methane, hydrogen, and carbonic acid, contrasting with fermentations of cellulose taking place at lower temperatures, where the chief product was butyric acid He further observed the remarkable phenomenon, that if the growth of these species was arrested by raising the temperature or by using antiseptics, it was possible to accumulate the cellobiose and glucose successively formed from the cellulose Kroulik found both aerobic and anaerobic species among the thermophilous bacteria, and described an aerobe which, when grown in a liquid extracted from fæces, etc, would form yellow specks on filter paper, which were seen to spread gradually and to attack the cellulose violently at an optimum temperature of 55°-60° C It forms oval spores, which afterwards lengthen to rather strong filaments, dividing into larger and smaller fractions In this aerobiosis formic acetic and butyric acid were produced, the only gas formed being carbon dioxide.

The species have found industrial application owing to the excellent work of Langwell and Lloyd Hind on a single *facultative anaerobic* species, capable of fermenting cellulose, which originated from a variety of plants and parts of plants The species was isolated from stable manure, and had its optimum temperature between 60° and 65° C In anaerobic cultures on glucose agar at 38° C were found two different types of colonies, some being round, semi-transparent, and of a yellowish colour, whereas others were opaque white with crinkled edge Both types, however, seem to belong to one and the same species, described as an immobile bacillus (0 4-4 μ), forming spores at the swollen end For nutriment it only requires ammonium salts, potash, and phosphates The fermentation products (alcohol, acetic, lactic, and butyric acid, carbonic acid, hydrogen, and methane) are present in variable proportions, depending on the nature of the medium and the conditions of fermentation—access of air, etc The fermentations were carried out at 68° C. on a large scale, and led to good practical results with common green plants that require only mechanical treatment, and dry vegetables requiring brief boiling with dilute sulphuric acid Under these conditions the species is able entirely to destroy cellulose, whereas heavily lignified cellulose, such as wood, calls for a fairly expensive treatment

These investigations have demonstrated that this group of bacteria include very different species, or probably rather varieties, fluctuating largely in their action according to external conditions. In course of time, when their life-history has been cleared up more completely, they are likely to acquire great importance in industry.

5. Alcohol-forming Bacteria.

Quite a number of bacteria produce alcohol amongst their products of fermentation The first known species was discovered by Fitz in a cold extract of hay, and was afterwards more exactly investigated by H Buchner, and described as *Bac Fitzianus* (Fig 26) It occurs both in coccus and bacillus forms. In a nutrient solution containing glycerine it ferments the latter, forming principally ethyl alcohol *Bac ethaceticus*, discovered by P Frankland in sheep dung, produces ethyl alcohol and acetic acid from glycerine, starch, saccharose, lactose, glucose, mannite, and arabinose *Bac pneumoniæ*, described by Friedlander, is not only a pathogenic organism, but also has the power of decomposing saccharine nutritive solutions, and forming ethyl alcohol and acetic

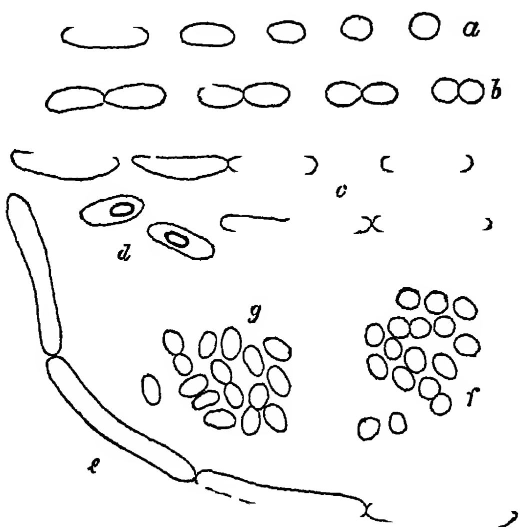


Fig 26—*Bacillus Fitzianus*, after H Buchner—*a*, *b*, *f*, *g*, *Coccus* forms and short rods, *c*, *e*, long rods, *d*, spore-bearing rods

acid In this connection may be mentioned a lactic acid bacterium found by Kruis and Rayman in sour yeast mash which produced ethyl alcohol as a by-product Duclaux's *Amylobacter ethylicus* has certain characteristics in common with *A butylicus*, and occurs along with the latter, but produces ethyl alcohol and acetic acid

Fitz found a species (*Bac butylicus*) in cow dung which produces considerable quantities of butyl alcohol by fermentation of glycerine. Fitz describes it as occurring in the form of motile rods 5 to 6 μ in length and 2 μ wide He developed it in a solution containing 1 part of potassium phosphate, 0 5 of magnesium phosphate, 2 of peptone, and 100 of glycerine in 2,000 of water, to which must be

added 20 parts of calcium carbonate, and he found about 8 per cent. of butyl alcohol in the fermented liquid *Bac orthobutylicus*, Grimbert, already described, also has the power of producing a considerable quantity of butyl alcohol, especially from glucose, when the nutritive liquid has an acid reaction, or when for any other reason the bacteria are in a feeble state In the same way Perdrix's *Bacille amylozyme* yields this alcohol on fermentation

Beijerinck's genus, *Granulobacter*, includes a series of bacteria producing butyl alcohol. We shall only attempt to describe a process used by him for the preparation of such species He introduces coarsely ground meal of husked corn, in successive portions, into boiling water until the mass has the consistency of a thick paste. The last addition should not be subjected to a temperature of 100° for more than a few seconds After rapid cooling, it is placed in an incubator at 35°-37° C The pure cultivation may be carried out in sweet-wort gelatine under anaerobic conditions. The predominant species form white non-liquefying colonies, with *Clostridium* forms and oval spores

Duclaux describes a facultative anaerobe, *Amylobacter butylicus*, obtained by infecting a potato mash with garden soil It exhibits the usual swollen sporogenous cells and the granulose reaction. It ferments starch, and produces butyl alcohol, butyric acid, and acetic acid A large amount of alcohol is readily produced when calcium carbonate is used to neutralise the acid formed during the fermentation of starch The same alcohol is produced by fermentation of saccharose (which is not inverted), maltose, lactose, glycerine, mannite, and calcium carbonate.

Bacteria also occur which produce amyl alcohol (fusel oil), to these belong Perdrix's *Bacille amylozyme*, producing minute quantities of this alcohol from potato starch A similar species was discovered by Pereire and Guignard, and H Pringsheim isolated another from potatoes It is still an open question how far the amyl alcohol produced during an impure alcohol fermentation is due entirely to the action of such bacteria According to Ehrlich's experiments, fusel oil may be obtained by the action of alcohol yeasts on two of the decomposition products of albumen; leucin and isoleucin

A group of bacteria of special practical importance are those which, besides alcohol, produce considerable amounts of *acetone*. Schardinger, as early as 1905, described one of these species, *Bacillus macerans* The name indicates that, like organisms active in the retting process, they have a marked power of dissolving vegetable cell agglomerations The species referred to, which is a facultative anaerobe, developed in mashed potatoes, after sterilising one hour a day for three consecutive days in a stream of steam and subsequently allowing to stand at 37° C, in mud mixed with retted flax It forms slender, very motile rods, which in the spore stage

lose motility, the oval spore is formed in the swollen end of the cell In sugar broth the spore is destroyed only by three hours' boiling On dextrose-peptone gelatine it develops colonies resembling white dots, but on potato a slimy covering One way in which acetone fermentation could be produced was by introducing a broth culture into mashed potato containing $CaCO_3$ at 37° C. Acetone and ethyl alcohol are formed in an approximate ratio of 1 . 4. The most favourable reaction for the development is believed by Euler to be $p_H = 6\ 8$ (*Biohem Zeitschr*, 128, 1922).

Among several other species which also seem to be related to the group *B amylobacter* as established by Bredemann, may be mentioned *B violarius acetonicus* described by Bréaudat, which likewise forms ethyl alcohol and acetone The species isolated by Fernbach and Northrop offer particular interest Fernbach discovered in 1910 one which yields acetone and butyl alcohol in the ratio of 1 2 or 1 2 5 from starchy foodstuffs, maize, potatoes, etc, when subjected to a special fermentation under strictly aseptic conditions The nutrient liquid is prepared from uncrushed maize in a dilution of 5 to 10 per cent, with an addition of sodium hydroxide. The fermentation is accompanied by the evolution of carbon dioxide and hydrogen * Gill suggests that the cultures which are to excite such fermentation should be previously grown in a mash made from rice and oat-meal, and the mash being afterwards pasteurised, to destroy the vegetative cells and sow only the spores Under given conditions the addition of acetic acid or an acetate to the fermenting liquid increases the acetone, while the addition of butyric acid increases the yield of butyl alcohol.

A species found by Northrop, Ashe, and Senior, *Bac acetoethylicus*, resembles *Bac macerans* morphologically, and was likewise isolated from sliced potato, sterilised for twenty minutes, and then placed in an incubator at the optimum temperature, 40°-43° C. The colonies developed on glucose-agar plates are described as round with smooth surface, irregular outline, and regular or undulating edge The spores can stand boiling at least 20 minutes. It is a facultative anaerobic species, and in contrast with Schardinger's bacillus it ferments lævulose and galactose under anaerobic conditions, in presence of ammonium salts It ferments pentoses, hexoses, and starch Fermentation can be best carried out when the media are adjusted to $p_H = 8$ to 9 The yields of acetone and ethyl alcohol obtained amount to 8 to 9 per cent and 14 to 20 per cent respectively The most suitable material on a commercial scale is maize. The addition of peptone or yeast extract, with slight access of air, increases the yield of acetone, while diminishing the yield of alcohol The maximum for a complete fermentation was 8 of maize to 100 of water

Good fermentations were obtained by Paterson, Fred and

* During the last ten years many thousands of tons of acetone and ethyl alcohol have been manufactured in England, France and America by the Fernbach process

Verhulst by the use of maize cobs (boiled with sulphuric acid), neutralising with milk of lime, pressing, or washing out the sugar, adding peptone, di-sodium phosphate, and calcium carbonate to the sugar solution, and partly filling the flasks with purified charcoal To insure a good development of acetone and alcohol, it is important to maintain an acid reaction of p_H 5 0 to 7 0 throughout the whole course of fermentation

Finally, among the different varieties of the *amylobacter* group, Thaysen isolated a growth which was specially active in producing acetone and butyl alcohol in the ratio of 1 2 from starch maltose and dextrose If sown in large quantity, it will also grow aerobically in a thick mash of maize or rice In the laboratory it was grown in a 5 per cent mash of maize flour, the fermentation being carried out in very tall glass tubes Spores can be isolated by heating to 90° C for a quarter of an hour and employed for fresh inoculations The culture is renewed by spores kept in sterilised sand or earth and cultured at 37°-39° C some time before use Industrial large-scale fermentations with this variety were completed in the shortest time at a temperature of 39°-40° C As in all such cases, it is necessary to control carefully the acidity throughout the fermentation.

6. Slime-forming Bacteria.

Among the various species of slime-forming bacteria there are several which are of peculiar interest in the fermentation industries, as they occur in wine, milk, beet juice, and fermenting wort, causing morbid changes By analogy, this slime formation, which usually consists of mucilaginous substances, may be regarded as a phenomenon closely related to the commonly occurring zooglœa formation of certain bacteria.

In his *Études sur la bière* (Plate 1, Fig 4) Pasteur described bead-like chains of spherical organisms, which render wine, beer, and wort so viscous that they can be drawn out into threads, this is caused by the formation of gum and mannite.

Kramer has described *Bacillus viscosus sacchari*, which in a short time converts neutral or slightly alkaline cane-sugar solution into a tough mass of a mucilaginous nature He isolated a *Bac viscosus vini* (2 to 6 μ long), which was cultivated in sterile **wine,** air being excluded Sound wines infected with this growth thickened in the course of six to eight weeks It grows best at 15°-18° C, and apparently cannot exist at such a comparatively low temperature as 30° C

A mannite fermentation is sometimes associated with the formation of slime in wine The motionless bacterium isolated by Gayon and Dubourg grows on the bottom as large zooglœa, and thrives only in saccharine solutions For the "fat-ferment" (*ferment de la*

graisse) observed by Kayser and Manceau, see the chapter dealing with the diseases of wine (Lactic bacteria)

Bacteria have been found in **bread** which produce a strong formation of slime, and in particular the "potato bacilli" appear to be active—*i.e*, varieties of *Bac mesentericus vulgatus* (*Bac panis viscosi*), described by Kratschmer and Niemitowicz, and by Uffelmann, Thomann, Vogel, and others As a consequence of the action of these bacteria the bread can be drawn out into long thin glutinous strings They occur in rye meal and multiply in presence of moisture They develop in bread if the spores survive the baking temperature, and the bread is stored in a warm place According to Migula, *Bac panis* (Vogel) occurs in long slender rods (4 to 7 μ), forming chains, which have a rapid movement, and possess a polar cilium They form oval spores, which survive the action of a current of steam at 100° C for fifteen minutes (in a potato culture) On gelatine plates the colonies form flat liquefied depressions With a magnification of 70, it appears as a colony having a yellowish-brown nucleus coarsely granulated, and delicate streamers in the gelatine On agar also the colonies form a nucleus with streamers The optimum is at 40°-42° C Kayser also found a *mesentericus* species in slimy bread The most efficaceous preventive of the growth of these disease-germs was found (as in similar cases) to be a sufficient lactic acidification of the bread

In **plant infusion** (*digitalis* leaves), Ritsert proved experimentally the presence of a *Bact gummosum* which brings about a mucilaginous formation of slime Its activity depends upon the sugar content of the liquid, and is greatly favoured by the presence of potassium and sodium acetate and yeast ash There is a rich formation of slime in 10 to 30 per cent nutrient cane-sugar solution, whereas none occurs in similar grape-sugar and milk-sugar solutions The species has a pronounced demand for oxygen, and the cells exhibit movement at certain stages It appears to occur both as rods and coccus forms, according to the composition and reaction of the substratum It liquefies alkaline gelatine In a stab-culture on agar it grows as a moist glistening whitish deposit, which forms two zones, the inner wrinkled and the outer smooth Brautigam isolated a *Micrococcus* from an infusion of *digitalis* leaves, which converted a nutrient sugar solution into a complete jelly. and made apple juice viscous In a similar infusion Happ found a slime-forming rod bacterium (*Bact gummosus*) 5 to 7 5 μ long, 0 6 to 2 μ wide It assumes spindle shapes in old cultures, and is sometimes motile On neutral gelatine it forms colonies with streamers, the gelatine is liquefied On potatoes it forms coccus-like involution forms Saccharose solution is absolutely necessary for the production of slime The optimum lies at 25°-30° C He also found a *Micrococcus gummosus*, which may be distinguished from Bräutigam's species by its fermentation products It forms yellowish colonies

on gelatine, but a colourless deposit on agar The optimum is at 15°-20° C This species may produce slime in saccharose and maltose solution Schardinger has undertaken a detailed enquiry into the products formed by a slime bacterium, one species of which was isolated from impure drinking water It is a very short, motionless, non-sporogenous bacterium which forms on gelatine a tough slimy film consisting of cells linked in long chains On saccharose or grape-sugar gelatine it forms slimy and "ropy" colonies of a greyish-white appearance, which when removed leave a depression in the gelatine In broth it forms slimy flakes, especially on the surface, and it also makes milk viscous In nutritive liquids containing saccharose, maltose, lactose, etc , it causes fermentation with evolution of hydrogen, and by fermentation of an 8 per cent saccharose solution, with inorganic salts and calcium carbonate to neutralise the acid, it forms lactic acid, acetic acid, ethyl alcohol, and succinic acid The optimum for slime formation is 20°-30° C It does not liquefy gelatine According to Schardinger, the species is related to Löffler's *Bac lactis pituitosi* A chemical examination of the slime formed by mass cultures from saccharose solutions, containing nutritive salts and calcium carbonate, shows that it chiefly consists of a carbohydrate which by oxidation with nitric acid forms mucic acid, and by boiling with hydrochloric acid produces optically active sugar As a slime can also be formed by bacteria in the absence of sugar, it should probably be regarded as a product of the swelling of bacterial membrane

As an example of one of the species producing a vigorous formation of slime in **milk** may be mentioned *Bac lactis viscosus*, found in water, and described by Adametz It forms a short, feebly-motile rod with a thick refractive capsule Its average dimensions (in milk cultures) are 1·5 μ long and 1 25 μ thick On glycerine-peptone-gelatine it forms whitish non-liquefying colonies with irregular jagged edges, which shows a bright opalescence in reflected light By inoculation in sterilised milk, the milk becomes viscid like honey in four to six weeks, and may be drawn out into long threads At the same time the fat globules of the milk disappear. Lactose is only attacked to a very slight extent by this species, whereas casein is greatly modified Slime is also formed in nutritive liquids free from carbohydrates It is believed to be a zooglœa formation

The *Coccus lactis viscosus* described by Gruber, which exerts a strongly slime-forming action on milk, generally occurs in *tetrads* and sarcina-like groups , grows best when air has no access On gelatine it forms under the surface small whitish, irregularly-shaped colonies, over which the gelatine is quickly liquefied On inoculation in milk, the reaction at 32°-34° C is first slightly alkaline, later acid , casein is thrown down and gradually peptonised , at room temperature the casein is not precipitated ,

but in every case the milk soon acquires a slimy consistency. In broth the growth is very feeble. In milk warmed up to 80° C the coccus was killed in two minutes.

Other related species have been described by Duclaux, Leichmann, Schmidt-Mühlheim, Loffler (*Bac. lact. pituitosi*, motionless rods which quickly divide into coccus-like cells, and on gelatine give white colonies with sharp or slightly dented edges), Weigmann (the coccus of "lange Wei" with nitrogenous slime), Emmerling, etc.

Emmerling has proved that *Bact. lactis aerogenes* forms a mucilage in lactose solutions possessing the properties of galactan, for by oxidation it may be transformed into mucic acid.

A species which apparently cannot be identified with any of the foregoing is described by Thom and Thaysen under the name of *Micrococcus mucofaciens*. It occurs as cocci, diplococci, and tetrads, frequently flattened on one side, diameter 0.8 to 1.6 μ, non-motil. Grows freely when air has free access. Optimum of growth about 33° C. On gelatine light-yellow colonies, which liquefy it very slowly, and cause ropiness; so also on agar. In broth this species forms a white sediment; but the liquid does not become ropy. Inoculated in milk at 22° and 35°, by rapid degrees the whole liquid becomes very ropy. In milk at 60° it is killed in thirty minutes. In a 1 per cent. lime-milk it is destroyed in the same time.

In **beer** also slime-forming bacteria occur. Thus H. Schroder (1885) found a *Micrococcus* in "ropy" Berlin "Weissbier," which was afterwards cultivated in a pure state by P. Lindner, who named it *Pediococcus viscosus*. The disease could be produced by adding pure cultures to sterilised "Weissbier" wort. On the other hand, this organism had no action on hopped beer-wort or low-fermentation beers. By the addition of tartaric acid the beer becomes normal. Schonfeld distinguished many species in long "Weissbier," and, in particular, found two typical kinds (*P. major* and *minor*). The optimum for the formation of slime lies between 20° and 26° C. These species form a considerable amount of acid, and impart to the beer a pleasant, acid-wine bouquet. In presence of larger quantities of alcohol the beer does not easily turn viscid, and the lactic acid present protects such beer from the disease.

These organisms grow well, according to Schonfeld, in ammoniacal yeast decoction. He proved that such species occur in horse urine.

A *Pediococcus* species was also detected by Schonfeld in lager-beer, which was acidified and rendered markedly slimy.

Zeidler (1890) isolated from lager-beer a slime-forming bacterium, microscopically resembling *B. aceti*. It forms in beer a slimy, stratified sediment, which on being poured out looks like the white

of an egg On wort-gelatine it forms round, yellowish colonies with rather smooth edges, it liquefies this, but not broth-gelatine In wort and beer it produces an abundant quantity of acetic acid In wort fermented by a mixture of this bacterium with pure yeast, no slime-formation occurred, not even after storage When the latter was infected after primary fermentation, a slimy deposit appeared in the bottles stopped up with cotton-wool and allowed to stand at 5° Réaumer, whereas those which had been corked up did not exhibit any slime-formation At 16° and 24° R, on the contrary, the beer in all cases became excessively slimy

In ropy Belgian beer, Van Laer found the cause of this disease to be small and very thin, sporogenous rods (1·6 to 2·4 μ long), which were partly isolated and partly united in pairs by means of a zoogloea-like substance When added to beer-wort, this first becomes turbid, and afterwards ropy Milk also turns slimy, and its lactose ferments On beef-broth gelatine these rods give concave colonies with concentric rings of different colours, streak cultures give broad, white bands, with a sinuous border, stab-cultures give a white stripe soon extending to the bottom of the glass, the gelatine forms fissures which become filled with the growth, while at the same time a speck is formed on the surface. Experiments carried out with pure cultures of this bacterium in beer-wort have shown that one and the same form includes many varieties, which have a somewhat different action on wort They are all included under the name *Bacillus viscosus* (I and II) If sterilised wort is infected with this bacterium, and alcoholic yeast added after the lapse of some hours, the liquid becomes viscous If the wort is infected with a mixture of absolutely pure yeast and bacteria, the disease will develop in a varying degree, according to the proportion of bacteria If, however, these are only added after the completion of the primary fermentation, the disease will not appear at all. The greater the proportion of nitrogenous matter in the liquid, the sooner it will become viscous, even liquids which do not contain sugar can be made ropy by these species When the nutritive liquid contains much sugar, the fungus develops very feebly, and in pure sugar solutions the phenomenon does not occur. A high content of acid greatly restricts the development of these bacteria.

Van Laer has since isolated a *Bac viscosus bruxellensis* which produces, in addition to slime, a peculiar disease called "bière à double face" It occurs in "spontaneously" fermented Belgian beers, Lambic, Faro, and Mars, and can be recognised by the fact that the beer looks clear in transmitted light, and milky in reflected light. It forms a long rod making a white tough film on beer-wort, which grows down into the liquid Subsequently the slime disappears, and the rods are then surrounded by a slimy envelope. On wort gelatine large, round, slimy, transparent colonies are

formed, with a yellow centre and with many zones The species restricts the activity of alcohol yeasts, and beer attacked by it is consequently poor in alcohol, and richer in extract than sound beer It forms lactic, acetic, and butyric acids

Kayser isolated from ropy beer a bacterium which appears as *cocci*, *diplococci*, and *tetrads*, size about 1 7 μ, forming whitish-yellow colonies on solid media Optimum temperature 25°-28° C It is a facultative anaerobe and grows readily on beer wort, yeast-water, sugared peptonised broth, and beer, all these liquids becoming ropy Besides the common sugars, it also attacks galactose, arabinose, dextrin, and mannite Secretes maltase Supports up to 6 per cent of alcohol, and forms slime even in liquids containing about 350 g of hops per hectolitre Like several slime-forming bacteria, it forms lactic acid and volatile acids Tests with various additions to beer, sterilised at low temperature, proved that this bacterium gave the most abundant yield of acid when asparagin was added Compared with the other slime-forming bacteria found in wine and cider and studied by Kayser, this species is distinguished by yielding considerably less volatile acids from maltose, saccharose, glucose, and lævulose, in proportion to non-volatile acids, and it also differs by forming alcohol only from lævulose, and not yielding mannitol from this sugar

Vandam found in English beers an aerobic *Bac viscosus* (III), which occurs as small rods, single or in chains, consisting of two, three, or more links, with spore-formation in the centre of the rods This bacillus develops best at about 30° C, and produces a slimy mass in brewers' wort, which under the microscope proves to consist of zooglœa formation After the lapse of some time the liquid has the consistency of albumen No gas is evolved, but the liquid acquires a peculiar odour On meat-juice gelatine and on wort-gelatine the growth develops freely The viscosity of the liquid does not seem to depend on the quantity of nitrogenous matter present, but on the other hand, the bacillus grows feebly in the absence of sugar. This species is incapable of producing disease in beer unless it is thriving well, and is introduced in large quantities into the wort before or during pitching Like the form discovered by van Laer, it ferments milk-sugar, and, according to Vandam, it is easy to detect it in yeast, even in traces, simply by introducing a sample of the latter into nutritive liquid containing milk-sugar, a growth of this species soon making its appearance in the upper part of the liquid

Brown and Morris mention a *Coccus* form which also seems to produce ropiness in English beer This species occurs as diplococci and tetrads, and gives yellow wax-like colonies on meat-juice gelatine The disease made its appearance in the beer after a lapse of six to eight weeks, but it was not usually possible to produce it by inoculation with pure cultures of the species in sterile

beer Close to the fermentation room there was a pork-butc premises, in which putrefying matter had accumulated; after had been removed and the soil dug and cleaned, the disease appeared

Fellowes also examined several English beers affected by disease, and prepared pure cultures of the bacteria present, by inoculation of the cultures in beer he did not succeed in paring a beer containing these organisms and showing a visc corresponding to that of the sample from which they came.

Heron undertook a thorough study of a slime-ferment w occurred in English beers, a very small coccus, which gradu elongates, and by contracting in the middle assumes the form dumb-bell The two ends may also expand in a direction at r angles to the first growth, and assume a similar shape. At a stage the species takes on the form of rosaries (zooglœa) The attacked loses its acid simultaneously with the formation of m lage, and acquires an unpleasant taste This species can produce slime in presence of yeast Beer may be protected aga its action by increasing its acidity and adding more hops species originates in malt dust, according to Heron

The bacteria causing ropiness in beer examined by Baker others, are described in the chapter on acetic acid bacteria.

The so-called frog-spawn fungus *Leuconostoc* (*Streptococ mesenterioides* was investigated by Cienkowski and van Tieg and subsequently by Zopf and Liesenberg (Fig 27) Both European form and the variety found by Winter in Java o spontaneously in beet-juice and in the molasses of the sugar fact and in molasses distilleries, in which they form large slimy ma ("frog-spawn") and multiply vigorously The fungus fo chains of cocci, alternate pairs of which are always more clo united In contrast to the observations of earlier workers, thought that certain of these cocci enclosed spores, Zopf fo that they present no differences morphologically or physiologica spore-formation could in no case be proved Consequently, analogy formerly assumed to exist between this fungus and algæ genus *Nostoc* (implied in the name *Leuconostoc*) falls throug

Under certain conditions the cells are surrounded by a str gelatinous sheath with a sharp outline (*Bb*, *Bc*, *C*), which in m of the above consists of a mucilaginous carbohydrate, *dext* This formation only takes place in the presence of cane and gra sugar, and not in solutions of milk-sugar, maltose, or dext Under the latter conditions, and in potato cultures, the spo develop distinctive forms, in which the gelatinous sheath is co pletely absent (*A*, *Ba*) The formation of jelly is a phenome depending also upon certain conditions of nutriment

Leuconostoc ferments grape-sugar, cane-sugar (after previ inversion), milk-sugar, maltose, and dextrin, with production

acid and gas. According to Owen it is only the unsheathed form that attacks sugar The acid proved to be lactic acid. Especially characteristic of this fungus is its power of resisting high temperatures, the younger growths possessing this power in a higher degree than older cultures It withstands gradual heating to 86°-87° C for a few minutes The optimum temperature for development lies between 30° and 35° C , the maximum at 40°-43° C It is also remarkable that both the growth and the fermentative activity of the fungus are favourably affected by the presence of considerable quantities of calcium chloride

The *Lactococcus dextranicus* described by Beijerinck is, according to Smit, merely a stage of development of the species just described.

Fig. 27 —*Leuconostoc mesenteroides*, Cienkowski (after Zopf) —*A*, Cell cluster of the sheathless variety, taken from a potato cultivation , *B*, series showing the development of a culture, grown in gelatine, free from sugar , *Ba*, sheathless , *Bb*, the same after 24 hours' growth in a solution of molasses, sheaths already seen but not strongly developed , *Bc*, after 48 hours' growth in molasses, the sheaths strongly developed and partly encased in each other , *C*, a small gelatinous mass from which the cells have been expelled

Pitoy, on the leaves of a *Eucalyptus*, discovered an apparently distinct species, *L dissiliens*, which in sugar solutions appears as a *Streptococcus*, forming a slimy matter and producing carbonic acid, but no alcohol. It attacks only directly fermentable sugar, and grows most readily in neutral or alkaline liquids

Cohn's *Ascococcus* (*Micrococcus*) *Billrothi*, the cells of which are

enveloped in a jelly, under certain conditions of nourishment, forms mucilaginous slime from sugar, according to Zopf The three following species may be classed along with the above — Glaser described a *Bact gelatinosum betæ* which produces slime in beet-juice and evolves gas It forms short motile rods, giving liquefying colonies on beet-juice gelatine At its optimum of 40°-45° C, it rapidly forms a gelatinous film on beet-juice, it does not, however, develop on molasses It inverts saccharose, and produces alcohol during fermentation The slime is of the same character as in *Leuconostoc* *Clostridium gelatinosum*, described by Laxa and Schone, is found in sugar factories, and forms a slime like that in *Leuconostoc* It appears as rods of varying length, which are motile in their earlier stages, and form spores in the middle of the swollen cells The optimum is at 40° C The species inverts saccharose, and thrives best with free access of air In soil where sugar-beet is cultivated it grows in great numbers Maassen has described a number of similar species under the general name of *Semiclostridium*, by which he wishes to express that the rods, especially when the quantity of oxygen is restricted, swell, at one end and in the middle, the ellipsoidal spores do not, however, develop in this swelling, but at the thin end of the cell; the young rods are motile The optimum for vegetative growth is about 45° C The spores are extraordinarily resistant, both to boiling and to antiseptics, and the organisms are widely distributed in the soil

S commune, isolated from filter press residues, forms a jelly only from saccharose, which is inverted by this species, and fermented with evolution of carbon dioxide It may be distinguished from *Leuconostoc* by the fact that the slime yields lævulose on hydrolysis, whilst *Leuconostoc* slime forms dextrose

Cobb describes a gum disease on the sugar-cane, causing the production of a slimy yellowish mass in the vascular bundles of the stem, filled with bacteria of a single species, *Bac vasculorum*, which, according to Cobb, produces the mucilage In the gummy runnings of the sugar cane, a short rod with cilia always occurs, according to Smith, who named it *Bac Sacchari* The "gummosis" of turnips and sugar beets, recognisable by drops of gum appearing on the cross sections, which acquire a black colour, is accompanied by a strong development of bacteria These gradually multiply, and entirely alter the character of the mass Busse (experiments on the inoculation of pure cultures into sound beets) proved that the short motile rod which forms slimy colonies both on gelatine and on slices of beet was the cause of the disease. It inverts saccharose

The **Ginger-beer Plant** has been examined both botanically and biologically by Professor Marshall Ward If this ferment is introduced into saccharine solutions containing ginger, it transforms

them into an acid, effervescing beverage, ginger-beer. When fresh, it forms solid, white, translucent lumps, of irregular shape, brittle like dried jelly, varying in size from that of a pin's head to that of a large plum. It induces an alcoholic fermentation in the sugar solution, which at the same time becomes viscous. Marshall Ward isolated the numerous micro-organisms existing in these lumps, and described a series of yeast-fungi, bacteria, and moulds, and of these, two organisms proved to be essentially concerned in the fermentation of ginger-beer. One is a *Saccharomyces* (bottom yeast), belonging to the ellipsoidal group of this genus, and probably originating from the ginger and brown sugar commonly used; Ward named it *Saccharomyces pyriformis*. It inverts cane-sugar, actively ferments the products, and forms a pasty white deposit at the bottom of the vessel. It yields spores on gypsum blocks in 40 to 50 hours at 25° C.; it also forms spores on gelatine. In hopped wort it induces a feeble fermentation, and forms a film on the surface containing many pear- and sausage-shaped cells.

The other essential organism, which is alway present, is a Schizomycete, *Bacterium vermiforme*, which, according to Professor Ward, emanates from ginger, and is active in the lactic acid fermentation. It is a peculiarly vermiform organism, enclosed in clear, swollen, gelatinous sheaths, and imprisoning the yeast cells in brain-like masses formed by its convolutions. It is the swollen sheaths of this organism which constitute the jelly-like matrix of the "plant." It also appears without sheaths, and in a great variety of shapes. The gelatinous sheaths are only developed when the saccharine liquid is acid, and free from oxygen.

A *Mycoderma* and a *Bacterium aceti* were also found.

Marshall Ward has proved experimentally that *Saccharomyces pyriformis* and *Bacterium vermiforme* are the only two essential species in the ginger-beer fermentation, since it was only by inducing a fermentation with these two species that he was able to produce an effect similar to that obtained when the ordinary ginger-beer plant is employed. But it is only when both species develop together in the liquid that they bring about this result, and his experiments indicate that the relations between the yeast and the bacterium are those of true symbiosis, because the yeast ferments more vigorously in presence of the bacterium than it does alone.

7. Bacteria with Inverting, Diastatic and Proteolytic Enzymes.

We have already mentioned a number of bacteria that owe their importance in the fermentation industry to enzymes. Some further examples are given in this section which possess other enzymes.

Invertase is present in the following, amongst others —

Bact (*Proteus*) *vulgaris*, one of the commonest putrefactive bacteria, forming short motile rods often grouped in rows, and also forming long filaments with spiral and spirulina forms

The various strains grow under aerobic as well as anaerobic conditions, and thrive on widely different media, at high and low temperatures On gelatine they form grey translucent colonies, which soon sink in the gelatine They occur commonly in putrid flesh

Bact. fluorescens liquefaciens, which occurs frequently in water, as well as in decomposing substances, and derives its name from a greenish fluorescent colour which it imparts to gelatine The gelatine is liquefied It forms straight and curved rods of medium size, consisting of two or more members

Bact Megatherium, found by de Bary on boiled cabbage leaves, is distinguished by its extraordinary size The rods may be 2·5 μ thick, they sub-divide into short cells It forms whitish, liquefying colonies on gelatine

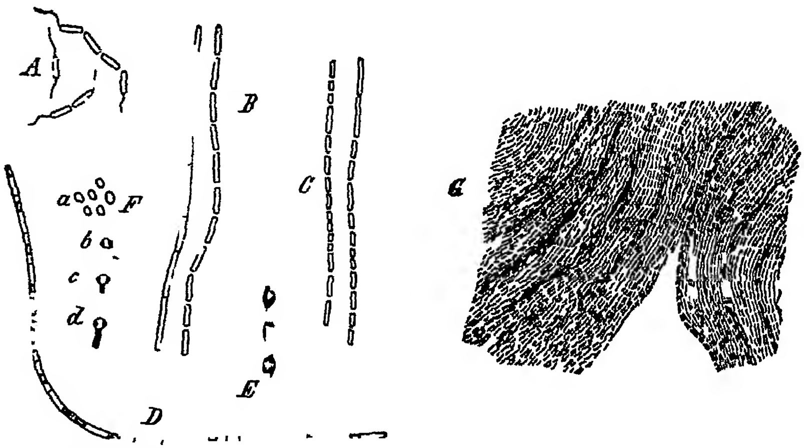


Fig 28—*Bacillus subtilis*—*A*, Cells with cilia; *B*, *C*, segmented threads, *D*, thread with spores, *E*, spores in swollen mother-cell, *F*, germination of spores, *G*, film on hay infusion

Fermi and Montesano found that *Bac Megatherium*, *Proteus vulgaris*, and *Bac fluorescens liquefaciens* in neutral broth, invert a 4 per cent. solution of saccharose Many of these bacteria, however, lose their power of inversion if the broth is rendered alkaline, whilst most of them are uninjured in slightly acid broth In broth without sugar, and in media containing no albumen, such bacteria produce invertase, thus almost all the species that were examined formed invertase in a nutritive salt solution containing glycerine. The invertase produced by these bacteria proves to be a soluble

enzyme, which is destroyed at temperatures differing according to the species, but it is always more resistant during its action on saccharose than in a dissolved state, it is very sensitive to acids and alkalies, and especially to organic acids and potash

According to Hansen, many species of bacteria of common occurrence in beer secrete inverting ferments Amongst these there is a group which exhibits an inverting action on a pure saccharose solution, but loses this property when yeast-water is added

Wortmann in 1882 began some experiments on the **diastatic action** of bacteria, and used for this purpose drops of bacterial cultures from rotten beans or potatoes He proved that species were present which can bring about the same changes in starch paste and in soluble starch as the diastase of the higher plants. The bacteria only react on starch when no other available carbohydrate is present (*e g*, sugar or tartaric acid) Krabbe showed that the presence of peptone increased the formation of diastase Fermi proved that this enzyme was present in different *Streptothrix* species, and found that the formation of diastase was prevented when the bacteria were cultivated on substrata free from albuminoids Pfeffer and Katz observed a rich formation of diastase in *Bac Megatherium*, by the addition of saccharose or maltose to the nutrient, the diastatic activity was considerably reduced Garbowski observed the enzyme in his detailed research on *Bac luteus* The reaction is brought out most strongly by inoculating an inorganic nutritive liquid mixed with starch solution

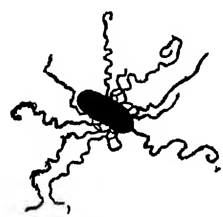


Fig 29 — *Bacillus subtilis* (after A Fischer) Cilia staining × 1,500

The ubiquitous *Hay bacillus*, *B subtilis*,* deserves special notice It secretes diastase in presence of peptone, contains an oxydase which yields *d*-fructose from mannite, and proteolytic enzymes.† The name includes, according to Kellermann's and Fawcett's extensive work, a considerable number of species or varieties As shown in the illustration, it forms short rods (often linked to long chains) and long filaments The rods carry numerous flagella and are motile The spores are oval and germinate at right angles to the longitudinal axis Optimum of the growth about 30° C, of spore-formation and germination 35°-38° C They are aerobic organisms, but will grow under favourable nutritive conditions even if traces of oxygen are present On gelatine they

* An abundant development of these organisms can be obtained by heating a filtered hay infusion in a stream of steam, for about 15 minutes The liquid will then contain chiefly spores of *B subtilis* and *B. mesentericus*

† The greatest proteolytic activity was found by Itano in a medium whose initial reaction was $P_H = 5\cdot42$ According to Swiatopelk-Zawadski, the proteolytic action of these bacteria is purely peptonising

form small whitish colonies, which gradually sink in the gelatine The stab culture is characterised by a straight column, devoid of ramifications Cultures on potatoes exhibit a whitish-yellow coating, which after some time becomes mealy Several species of this group seem to be active in plant diseases. According to Adr. J. Brown, *B. subtilis* will not grow in wort or beer of normal acidity. But N. van Laer found a similar species, which grows in beer and unhopped wort, having an injurious influence on the flavour and keeping properties of the beer It appears to flourish under both aerobic and anaerobic conditions. The spores, frequently found on barley and malt, will withstand two hours' boiling in unhopped wort, under other conditions they are even said to survive a five hours' boiling Lemoigne showed that certain species of the group play a prominent part in the purification of sewage

Bac vulgaris and *Bac. prodigiosus* are amongst the organisms containing **proteolytic or peptonising enzymes.** The latter, which belongs to the group of colour-forming bacteria (the "Bleeding Host") forms very short motile rods in weakly alkaline substratum, but longer rods and filaments in weak tartaric solutions. To these belong the *B. mesentericus vulgatus* described in the chapter on slime-forming bacteria This specific name includes a group of so-called *potato-bacteria*, which develop when a potato that has been sliced, but not washed, is placed in water at about 38° C The bacteria must therefore exist in the soil adhering to the potatoes. An aerobic culture of such an organism forms on gelatine, greyish, slightly fluted colonies, which soon sink in the gelatine If grown on potato, it forms very peculiar flutings, twisted and entangled somewhat like the coils of the intestines, eventually covering the whole surface of the potato with a slimy coat These bacteria appear as slender, motile rods, often linked as filaments. Spores slightly oval. Some species also secrete a starch-dissolving enzyme, which is utilised on a large scale in the amylo-process (Boidin, Effront), where both this and the proteolytic enzyme are employed in the treatment of the raw material The enzyme liquefies the starch and promptly converts amylo- and erythro-dextrins into achroo-dextrins, but its saccharifying power is less than that of common diastase The enzyme is isolated by cultivating the bacteria on an alkaline, nitrogenous medium devoid of starch, with abundant access of air. After washing, the enzyme is precipitated by alcohol and ammonium sulphate, and concentrated *in vacuo* (*Compt. rend., Ac. d sc.,* 164, 1917.) These albuminoid-digesting bacteria play an important part in nature, in the degradation and successive synthesis of organic bodies. The anaerobic species appear to be specially active; for example, *Bac. putrificus*, which forms long motile rods, with spore-formation at their swollen ends

The putrefactive bacteria occurring in water are apt to develop in beers containing a low percentage of extract, probably because of the low acidity of such beers The development of these germs may be arrested by acidifying the wort with lactic acid Decarbonating of the water, by treatment with lime-water in the cold, may also be of value, partly because the water is simultaneously purified biologically.

9 Sarcina.

The name *Sarcina* is given to spherical bacteria (*Cocci*), which are commonly non-motile, and divide in all three planes Under favourable conditions of growth, and especially in liquids, the cells formed by division may remain clumped together, caught in the slime secreted by the cells, and thus, more or less cubical groups are constituted, which sometimes bear a certain resemblance to corded bales of cotton On solid substrata, on the contrary, many of these species break down rapidly into single cells, or remain grouped in clusters of two or four The harmful kinds occurring in beer, which belong to this group of bacteria, are really only known with division in two planes, and commonly appear as diplo- and tetracocci, whilst larger clusters are composed of irregularly-massed cells Until something definite is known about them, the species must, therefore, be classed in the group which divides in two planes (*Pediococcus*, *Micrococcus*, and *Merismopedia*) It is clear, from a large amount of research, that these bacteria display extraordinary variability, both morphologically and physiologically.

The many species of *Sarcina* that have been described, give variously coloured colonies on gelatine White or greyish colonies are formed by *S alutacea*, isolated by Gruber from leaven, which liquefies gelatine, this is also the case with Lindner's *S candida*, found in the water reservoir of a brewery. Yellow colonies are formed by the widely distributed *S flava*, which has been detected in leaven, beer, and elsewhere It forms both regular packets and irregular masses of cells. On gelatine, it gives small round colonies, which gradually liquefy the gelatine, and on hay infusion, it forms a film with a strong development of regular bundles. *S. aurantiaca* forms on gelatine orange-yellow, liquefying colonies but develops typical sarcina only in hay infusion and plant decoctions. It gives a dark, bluish-green colouration with sulphuric acid *S. casei*, discovered by Adametz in cheese, forms pale yellow, liquefying colonies with concentric rings and coagulates milk. Adametz also found *S. butyrica* in cheese, it forms a yellowish-white colony on the surface of stab-cultures in potato-gelatine, but dark liquefying colonies in plate-cultures *S lutea* always forms regular packets, and gives, on gelatine, lemon-yellow, non- (or only feebly) liquefying colonies Brown colonies on gelatine are given by *S. acidificans*,

discovered by Adametz in cheese It develops a yellow colony in stab-cultures on agar It precipitates casein from milk A dark brownish-yellow colour is developed by *S fusca*, discovered by Gruber in flour Among varieties exhibiting a red growth, are *S rubra*, giving glistening red colonies on the surface of gelatine, and slowly liquefying it The colouring matter is insoluble in alcohol *S rosacea*, occurring in air and water, usually forms irregular masses of cells in malt extract, but develops typical sarcina in a hay decoction, which are enveloped in brownish slime. On a neutral malt-extract-gelatine, it forms a reddish deposit with a dry surface The colouring matter is soluble in warm alcohol. *S maxima*, discovered by Lindner, which develops in a malt mash at 40° to 45° C, has cells of 3 to 4 μ diameter

S mobilis, isolated by Wolff from milk, is distinguished from each of the above by having motile cells It shows the typical form both in liquids and on solid substrata, liquefies gelatine, and forms yellow colonies on whey gelatine and agar

In the fermentation industry, sarcina-like organisms occur, in addition to those already mentioned in section 6, especially in low-fermentation lager beer, where they may develop during the secondary fermentation Pasteur described and depicted the *diplococcus* form, he noted that beer contaminated with such bacteria assumes a disagreeable flavour and odour At a later date, they were depicted by E C Hansen under the name *Sarcina* (Fig 30) He found them in many parts of the brewery plant. Balcke gave them the name *Pediococcus cerevisiæ*.

Fig 30—Sarcina

Other workers have since failed to isolate typical sarcina from diseased beer, and have only detected irregularly massed cells The name "*Sarcina* disease" is best retained, as definite conceptions are associated with it

Lindner has described a number of *Sarcina* species in pure culture, and, amongst them, one which occurs in diseased lager beer, which he named after Balcke, *Pediococcus cerevisiæ* By inoculating pure cultures it proved impossible to reproduce the unpleasant flavour and odour of the beer, only turbidity ensued In later experiments, Lindner occasionally succeeded in reproducing the characteristic appearance in beer by introducing yeast, which had been inoculated with a *Sarcina* isolated from the diseased beer On the other hand, A Petersen observed a case where a growth of these organisms had developed in beer without affecting either its flavour or odour

A Reichard isolated from low-fermentation beer a *Pediococcus sarcinæformis*, which developed freely in sweet wort or sterile beer, but not in pasteurised beer This species developed best with limited access of air In fermentation tests turbidity or peculiar changes of taste only ensued in a minority of cases. After many

experiments, he arrived at the conclusion that these contrary results were due, partly to the condition of the various cultures of the *Sarcina*, partly to the manner in which the fermentation took place In quiet fermentation in a lager cask, the growth remained at the bottom and the bacteria did not exert any appreciable influence on the liquid, whereas, in the case of a vigorous secondary fermentation, they were carried up with the bubbles of carbon dioxide, after which the disease manifested itself Rousing the beer may, therefore, be injurious in such cases Addition of hops to lager beer exerts a retarding influence on these organisms, as on the majority of bacteria occurring in beer

Two species have been described by N H Claussen, which were isolated after he had suppressed the growth of other organisms occurring in beer by a slight addition of acid ammonium fluoride. Beer cultures were allowed to develop in hopped wort and in pasteurised beer, and after inoculated in fresh beer, brought about the characteristic disease phenomena Both species grow in the usual nutritive liquids when either neutral or slightly acid, whereas a minute quantity of free alkali restricts their growth The most favourable temperature for growth is 23°-24° C Neither liquefies gelatine They grow in wort, both when oxygen is fully excluded and in presence of the normal atmosphere The one, *P damnosus*, usually imparts an unpleasant odour and flavour to beer, but only forms a slight deposit in the liquid, the other, *P. perniciosus*, causes a turbidity in the liquid, in addition to deterioration of flavour and odour Schonfeld has isolated species from diseased beers with the help of sweet wort gelatine, and especially on dry-yeast gelatine He found species that are dangerous to large beer, imparting the *Sarcina* odour and objectionable flavour, as well as turbidity, only produce a comparatively minute quantity of acid in sweet wort, and give a peculiar odour slightly resembling honey, for this reason he gave the group the common name of *P odoris mellisimilis* (he assumed that the group is identical with Claussen's *P perniciosus*) In contrast with this, a group of species exists which occur in lager beer and "Weissbier," producing a large quantity of acid in sweet wort, and turbidity (according to Schonfeld), but not the pronounced *Sarcina*-odour In sweet wort they give a pleasant but sourish odour and flavour They are grouped together under the name *P acedulefaciens* Other varieties have been described by Schonfeld, giving a red colour to lager beer. By inoculating a pure culture of a species producing strong turbidity, into pasteurised beer, which is allowed to stand, he showed that in most cases, only a sedimentary growth developed, but if, on the contrary, carbon dioxide is passed through the beer, freely-swimming bacteria develop which produce turbidity This observation agrees with those made by Reichard in practice The slime formation due to certain *Sarcina* and formerly observed

only in "white beer" (Weissbier) could also be produced by Schonfeld in lager beer, by passing carbon dioxide through the beer previously seeded with cultures from wort

In English top-fermentation beer Heron discovered a minute *Pediococcus* occurring for the most part in pairs It can be grown on neutral or slightly acid but not on alkaline wort-gelatine, where it forms colonies of a peculiar yellowish-green lustre, on wort it forms a thin greasy film of a distinct bluish appearance. In beer it forms a slight greyness some days after racking or bottling, which gradually developed to a thick cloudiness, making the beer unsaleable It had no influence on the flavour or odour of the beer. It developed only in mild beers and in pale ales with a comparatively small amount of hops, and further experiments proved that **hops** exercise a strong influence on its development, more particularly the amount of hops used in the copper. As sources of infection, Heron detected malt dust in one brewery, an effluent conveying spent yeast in another

From a number of individual observations of the associated conditions, the following conclusions may be drawn —Species may be isolated from yeast and from lager beer, capable of development, which appear to be incapable of exciting any disease whatsoever in the latter Amongst true disease species, a given organism appears to be unable to produce the specific disease under all conditions, even when these are favourable for cell-reproduction In the light of our present experience, it is reasonable to assume that this is caused by the condition of the liquid at the time when bacterial contamination took place Thus it has been asserted that wort showing deficient saccharification offers a favourable field for the deleterious activity of the organism

Both typical *Sarcina* and *Micrococci* (*Pediococci*) are widely distributed in nature, and may easily be recognised by the use of the usual liquids and gelatines Certain materials, such as horse urine and dung, appear to be particularly rich in pronounced *Sarcina* species Their presence can easily be verified also in malt- and malt dust It has not proved possible as yet to determine the natural habitat of beer *Sarcinæ* One reason is that such species cannot be distinguished from others that do not attack the liquor by an ordinary micro-biological analysis. The only accepted conclusions are —(*a*) That all true beer *Sarcinæ* that have been exactly investigated, cannot thrive in alkaline substrata (ammoniacal fluids or gelatine), (*b*) that they form whitish masses in streak cultures, and on the surface of stab-cultures, (*c*) that they are to be classed as facultative anaerobes, (*d*) that specially favourable conditions for development are to be found in badly saccharified wort and, according to Miskowsky, in malt extract with a high content of dissolved albuminoids (especially albumoses and peptones); in such a liquid they may remain unaltered for a long

time, and (e) that, like many other bacteria, they appear to be checked by excess of hop constituents

It follows, that it is impossible to distinguish by any general test, whether *Sarcina*-like bacteria in yeast or beer are able to produce disease in beer To answer this question, we must proceed experimentally—a difficult and tedious investigation. It would, however, obviously be foolish to neglect the usual test for *Sarcina*-like bacteria in yeast and beer, for if observed, there is always the possibility that dangerous species may be present It has been thoroughly established by experience that, in the early stages of fermentation, even a feeble growth of such bacteria may prove dangerous, and the present problem is to provide means whereby the analyst may detect traces of these organisms. Such a means is Claussen's method for treatment of yeast with minute quantities of acid ammonium fluoride which checks the growth of the yeast cells, so that subsequent inoculation in wort-gelatine will usually give a growth of *Sarcina* colonies The liquid adopted by Bettges and Heller may also be used, it consists of sweet wort completely fermented by the addition of yeast, starch is then added, and after clearing, it is neutralised with ammonia, and diluted to an alcohol content of 4 per cent The liquid is inoculated with a sample and development observed in the sealed preparation It will be found that the bacteria consist essentially of *Sarcinæ*

In the author's laboratory, for many years past, an addition of neutral yeast water (preserved in flasks with an excess of calcium carbonate) is made to the sample taken at the end of the principal fermentation After two days' standing, the *Sarcinæ* present will have multiplied sufficiently to be easily recognisable under the microscope Until further research has shown whether the habitat of these germs lies inside or outside the plant, efforts must be directed, in practice, to discover their habitat within the plant We must bear in mind the limits of our present knowledge, and we must not forget that direct observation of *Sarcina-like* germs in the plant itself (for example in the vats) is of greater consequence than the observation of numerous germs of similar microscopic appearance, by the help of plate-cultures, in the surrounding atmosphere or in the water supply By a properly organised system of disinfection, and often without application of antiseptics, such growths may be entirely suppressed. The impression that the beer *Sarcinæ* described in this section cannot be fully excluded is entirely erroneous, an impression arising from the extreme difficulty in distinguishing between certain organisms of this numerous group occurring in air and the true disease species

Research on bright wines in the author's laboratory have frequently brought to light vigorous growths of *Sarcina*, while the wine acquired a peculiar odour, which resembles, to a

arkable extent, the odour and taste of beers in which they ır

10 The Fermentation of Tobacco.

During the fermentation which dried tobacco leaves undergo, erous organisms are present, and it was naturally assumed they play some part in the successive degradation of the material During fermentation the temperature gradually , and attempts are made in various ways to limit the temture to about 50° C. The effect of fermentation is that aromatic es are produced in the leaves, and part of the nicotine, according Behrens, simultaneously disappears Suchsland was the first ivestigate the micro-organisms present in fermenting tobacco. attempted to improve its quality by inoculating with pure ires of selected species of bacteria. Nothing further has been ished regarding these species More recently, Behrens, Vern-, Koning, and others have described some of the vast number pecies that are present, and Koning found, by parallel experits, that inoculation with certain pure cultures selected from enting tobacco, partly aerobic, but chiefly facultative anaerobic, cised a favourable influence on the aroma and flavour of the cco In the same way the after-fermentation, which takes when the leaves are packed together, appears to be due to action of micro-organisms The contrary view has been exsed by O Loew, who attaches no importance to micro-organisms ie fermentation, but seeks the active causes in the oxidising mes, which he proved to exist in the leaves. H Jensen, as as Splendore, found that leaves which had been heated in a nt of steam (90°-100° C) showed every sign of a good fer-ation, and that this was not prevented by treatment of the s with mercuric chloride, formol, and chloroform, which would inly appear to confirm Loew's conclusion. Behrens bases direct observations his belief that micro-organisms do ince the course of the fermentation, a view, the correctness hich is rendered more probable, by Schloesing's results on the entation of snuff tobacco Experiments undertaken in the or's laboratory with parallel fermentations of both American African tobacco, led to the conclusion, especially when facultaanaerobes were employed, that certain species do play a part termining both the aroma and flavour of tobacco

11 Iron and Sulphur Bacteria Nitrifying Bacteria.

he bacteria described in this section are of particular interest, ise they possess the property of oxidising inorganic subes

ı the microscopical examination of water, we often meet with

characteristic forms of *Crenothrix Kuhniana* (Fig 31), or spring pest, described by Cohn and Zopf.

The organism occurs in all water containing organic matter, and sometimes multiplies to such an extent as to render the water unfit for use. Thus, according to Zopf, serious calamities have been caused by this fungus, in the water supplies of Berlin, Lille and certain Russian cities In consequence of its power of

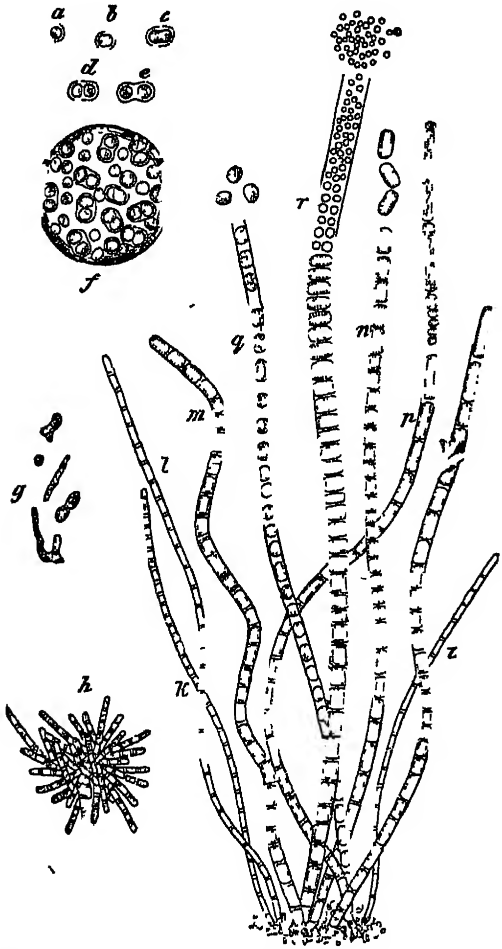


Fig 31 —*Crenothrix Kuhniana* (after Zopf).—*a-e* (600 1), Cocci in different stages of division, *f* (600 1), small, round cocci-zoogloea, *g* (natural size), zoogloea, *h* (600 : 1), colony of short filaments composed of rod-like cells, derived from the germination of a small collection of cocci, *i-r*, filaments, partly straight, partly spirally curved (*l*, *m*), of very varying thickness, with more or less pronounced contrast between base and apex, and different stages of division of their members and sheaths, the sheathed filament *r* shows short rods at the base, which further up are divided into small cylindrical joints, at the apex the cocci are seen arising from the longitudinal divisions of the cylindrical discs

storing iron compounds within its walls, it forms red or brown flakes in water Its various forms are very beautiful, it occurs as motionless cocci or gonidia (*a-f*), which by division and formation of viscous matter form zoogloea (*g*), these cocci frequently grow to articulated filaments, which are provided with distinct sheaths (*h*, *i-r*), they then increase in thickness towards their free end, and, when they reach a certain age, divide within the sheath into smaller fractions, which become round and issue either as rods, macro- or micrococci

Leptothrix ochracea is a widely distributed iron bacterium with colourless cylindrical cells, connected in threads and surrounded by a sheath, which is at first thin and colourless, but afterwards, by accretion of hydrated oxide of iron, assumes a yellow or brown colour Oval and motionless gonidia develop in the threads The empty sheaths may form large yellowish-brown deposits in water containing iron

Cladothrix (*Sphærotilus*) *dichotoma* is also of frequent occurrence. Its cells are surrounded by a similar thin sheath. By displacement of single rods in a filament, false branching takes place. The rods are finally set free, and are then provided with cilia, with which they swim about until they settle down and expand into new threads.

Iron bacteria are commonly found in water containing soluble basic ferrous carbonate. According to Winogradsky this salt is oxidised by the bacteria and ferric oxide is deposited on the sheath The great deposits of iron ochre found in nature may probably be partially accounted for by the activity of such bacteria. According to Molisch and others, they can also assimilate considerable quantities of manganese

Sulphur bacteria occurring in water, many of which produce a red colouring matter, have been described by Cohn, Warming, Engler, and especially Winogradsky Under the microscope they are distinguished by the roundish bodies they contain, strongly refractive to light, and consisting of pure sulphur. They are aerobic, and occur especially in waters containing sulphuretted hydrogen This substance is oxidised by the bacteria, and the sulphur split off is stored in the cells. Among the thread-like species, *Beggiatoa alba* may be mentioned It occurs in cylindrical filaments without sheaths, which have a crawling motion, rotate round their longer axis, and swing from either end. They may expand to a great length. The threads divide by means of cross sections, and if no sulphuretted hydrogen is present they break up into single pieces, and gradually die off.

A vital function is performed in nature by bacteria which convert ammoniacal salts into nitrates, they are highly important for the nutrition of plants Schloesing and Muntz first described them; their observations were confirmed by Winogradsky, who

made use of pure cultures. Among these nitrifying bacteria, as they are termed, there are some which oxidise ammonia into nitrous acid (nitrifying organisms), which is converted by other species into nitric acid (oxidising organisms). As stated in an earlier section, these bacteria possess the power of living without any organic food; in laboratory culture experiments they are grown in solutions of inorganic compounds They form cocci and short rods, aerobic, motile, or non-motile, they can grow in the dark, although they assimilate carbon dioxide from the air Again, saltpetre may be attacked by the denitrifying bacteria, which are capable of decomposing saltpetre in presence of organic matter with evolution of free nitrogen The nitrifying bacteria also cause the efflorescence of nitre from walls, which often brings about the decay of brickwork, snow-like masses of calcium nitrate being detached This evil can be remedied by means of antiseptics

CHAPTER IV.

MOULDS.

CERTAIN moulds as well as bacteria are of industrial importance, and have found application in the form of properly selected pure cultures. On the other hand, a large number of species are known that produce objectionable diseases in the different branches of the fermentation industry. They select as their habitat the vessels, tools, green malt, and quiescent masses of yeast, especially top-fermentation yeast. A close examination of mould growths, taken from the ceiling or walls of a fermenting room or from the lip of a vessel, soon shows that they scarcely ever consist of an unmixed growth of moulds Amongst the mycelia, bacteria and yeast-like cells can almost always be found. The hyphæ of the mould plant project outwards, carrying foreign germs with them, and these, in their exposed situation, are easily swept off either by air currents or by workmen. All kinds of micro-organisms occur on the raw material during the malting process If moulds are usually considered to be the worst enemies, it is due to the fact that they are visible to the naked eye, and so attract special attention If we judge by numbers, bacteria must certainly take the first place, for they are always present in great numbers on green malt It may even be considered doubtful whether the greatest influence on the product is due to the moulds (*Penicillium*, *Aspergillus*, etc.), when these are met with in a state of vigorous development on malt, or whether it is not far more probable that the numerous organisms accompanying them play the most important rôle It is doubtful whether the so-called "mouldy" smell of beer is caused by moulds. Observations made in the author's laboratory point rather to the action of bacteria In distilleries and yeast factories, on the other hand, moulds have been known to appear even during fermentation. Growths of *Oidium*, *Dematium*, etc, are, for example, found on the surface of the yeast layer in the vat, and the yeast-like cells which are produced by these fungi (and by *Mycoderma*), which bear a striking resemblance to true yeast cells, can frequently be observed multiplying in the upper yeast layer. They may be skimmed off along with the yeast, and thus the author has often found a fine white deposit on the surface of pressed yeast, which most frequently consists of a mould mycelium, generally belonging to the genera named above. It is quite possible that when these plants form a dense layer on the surface of the yeast-mass, they

retain by respiration, a portion of the free oxygen which is necessary to enable the quiescent yeast to remain alive As shown by Henneberg and Schnell, various Oidium species attack inert (pressed) yeast and kill the cells, they break down the membrane and degrade the albuminoids to free ammonia

All our experience suggests that a growth of mould nearly always indicates that other organisms of a more injurious and more active character are developing It is, therefore, of great importance that the walls of the fermenting rooms should be smooth, this is effected with the greatest certainty by employing the enamel paint now so popular.

The moulds are of real significance in the dairy industry The experience of recent years has shown that amongst the great diversity of forms which make their appearance in this industry, some of which are of the nature of unbidden guests, many contribute in no small measure to the improvement of the quality of cheese if proper means are adopted

Amongst other useful organisms may be mentioned *Aspergillus oryzæ* from Japan, which, on account of its powerful diastatic enzyme, is used for the manufacture of sugar from starch; *Amylomyces*, introduced from Indochina, and employed in European distilleries, *Citromyces* and *Aspergillus niger*, used in the preparation of citric acid from grape-sugar

The moulds, many of which represent stages in the life history of higher fungi, occur as vigorous growths easily visible to the naked eye Each species has a characteristic appearance, which is due, not only to its structure, but also to the colour which it affects at some particular stage in its development, varying in shade from the purest white to the deepest of colours

The individual cells of which the body of the mould plant is built up, consist of a cell-wall or membrane, together with the cell-contents, which consist essentially of protoplasm, vacuoles, and various contents, of which the most important is the cell-nucleus The **membrane** is composed of a substance known as fungus-cellulose, differing, as a rule, in its chemical reaction from the cellulose of higher plants. Both inner and outer surfaces are subject to gradual thickening, and with increasing age become impregnated with deposits of colouring matter, and incrusted with crystals, especially of calcium oxalate.

The **protoplasm** (or Cytoplasm) consists of a homogeneous viscid substance packed with minute granules. This constituent of the cell determines the growth, and a part of it forms a thin layer lining the inner surface of the cell-wall The protoplasm in lining cells is in constant motion; in certain cases (*e g*, the young sporangiophores of species of *Mucor*), the motion is sufficiently active to permit of its observation through the microscope In very young cells the protoplasm occupies the entire cell space

Later on **vacuoles** appear, and at the same time different kinds of corpuscles, amongst which the "**crystalloids**" may be cited, consisting of albuminoid substances, which may perhaps be regarded as products of secretion There are also the widely distributed **fatty oils** and fats, which are especially abundant in the reproductive organs and the resting cells A very important part of the protoplasmic contents is the **nucleus**, a small spherical body, which, by addition of a suitable stain, becomes very prominent, and under the microscope, may frequently be seen to include an inner and more highly coloured portion, which is called a nucleolus. By special methods of staining, it has been shown that the nuclei are capable of division and of fusion, processes which are directly connected with different stages of development which the vegetative and reproductive organs of the fungus pass through Much work on this subject has been done by Moreau (*Mucorinæ* and other *Thallophytes*) We shall return to this subject when we come to a description of yeasts The cells form the *mycelium* of the fungus This is composed of branched or unbranched filaments (*Hyphæ*), usually provided with transverse walls, the region of growth being always at the apex Outgrowths may arise from older cells which expand to form lateral branches Transverse cell-walls are usually absent in the mycelium of fungi belonging to the *Mucor* species. Thus the whole of the vegetative portion of the mycelium of these fungi, with its intricate network of branches, consists of a single cell Amongst members of this genus a second form is known Under special conditions, the submerged portion of the mycelium may divide into separate cells, which split off, become rotund, and give rise to protuberances which go through the same cycle of growth. This is the so-called **spherical yeast**, which grows in the same way as true yeast The same variation has been found to occur in many other moulds · there exist, moreover, forms exhibiting every intermediate stage down to those in which the mycelium is almost entirely suppressed, and the budding forms predominate

Many moulds form peculiar resting-organs; the walls are thickened and the mycelia closely packed together, surrounded by a dark, sometimes felted pseudo-cortex, formed by the outer hyphæ In this way small hard bodies, known as *Sclerotia*, are formed These are packed with stores of reserve foodstuff, and may retain their vitality for a lengthy period, thus ensuring the maintenance of the species during conditions unfavourable to growth

Passing on to the manner of reproduction of the moulds, we are brought face to face with the remarkable fact that these organisms, although occupying so lowly a position in the vegetable kingdom, possess in many cases, not one, but several entirely different methods of reproduction. Every stage of complexity

may be met with, from the simplest forms of reproductive organs to the most highly developed In *Oidium lactis*, reproduction is effected without the aid of any specialised organs The filaments of mycelium divide simply by means of transverse walls into short cylindrical pieces, so that the whole plant is finally transformed into numerous "oidia," each one capable of giving rise to a new individual

The reproductive bodies are termed **spores.** Of these, the simplest kind are the **conidia**, which are formed by constriction from one or more of the mycelial hyphæ, termed the conidiophores. This constriction may take place in two ways In the first, a new growth develops under the conidium first formed at the end of the thread, the new piece swells out to form a new conidium, and so development continues, successive spores being produced basipetally—*i e*, towards the base of the filament This is the method of spore-formation in *Penicillium* In the second case, the spore first formed at the end of the thread expands at its upper end, and is constricted to form a new conidium, the development proceeding in this way from the base of the thread upwards (basifugal) The conidia may, however, also develop spores laterally, a development similar to the budding of yeast cells This has been observed in *Cladosporium* The conidiophore may be branched, or may assume a still more complex structure, as in *Penicillium*, where numerous and minute conidia are linked in long chains, each at the tip of an individual branch The formation of conidia is usually arrested by acidifying the nutritive medium An altogether different kind of spore-formation is that which results in the production of **zygospores**, such as are met with in *Mucor*, and described later in detail The remarkable feature of this kind of spore-formation is that it is brought about by fusing together two cells, between which there appears to exist a definite distinction of sex In contrast to these, we find other spores produced in the *interior* of certain cells Here, again, *Mucor* serves as an example, where each mycelium carries a **sporangium,** in the interior of which a large number of oval or angular spores are formed round a column (columella), liberated by rupture of the wall

Quite a different variety of internal spore is met with in such genera as *Aspergillus* Here a small, definite number of spores are produced in a tubular cell (**ascus**) In many fungi, a large number of these spore tubes may occur combined to form fruit-like clusters, as in *Aspergillus*

The formation of fructification corpuscles was observed by Bezssonoff in *Penicillium glaucum* and several special of *Aspergillus*, when cultivated on media containing high percentages of cane-sugar (*e g.*, 42 to 48 per cent, dissolved in an aqueous solution of $CaCl_2$, $MgSO_4$, $NH_4H_2PO_4$, KNO_3, and $FeCl_3$, with or without

gelatine) When cultivated thus, the plasma of the cells exhibits a fine granular structure Bezssonoff asserts that, by his procedure, the development of the sexual plasma is brought about or assisted.

When ripe, the spore is capable of germinating An example of the development of a *Penicillium* conidium is given in Fig 33 The minute spherical cell first absorbs water and its volume soon swells to many times the original size The cell-wall then commences to bulge at a given point, the outgrowth gradually lengthening into a thread-like process, which is soon delimited from the mother cell by a transverse wall The growth of the filament continues, branches shoot out, and new transverse walls are formed Where the spore possesses a stout outer coat, as in the zygospore of *Mucor* (Fig 35), it is ruptured by the pressure exerted by the contiguous inner coat which grows out to form the germinal hypha

The spores of fungi are more resistant than the mycelium. E C Hansen has shown, for instance, that the spores of a species of *Penicillium* were capable of germination after having been kept dry for twenty-one years According to the same author, a crop of *Aspergillus glaucus* was obtained from dried spores after the lapse of sixteen years The spores of many species can endure very high temperatures, especially in the dry state Thus dry *Penicillium* spores withstood a temperature of 120° C, but when damp they were killed at 100° C The remarkable power of resistance to high temperatures, which range far beyond that at which albumen coagulates, has been explained by assuming that the albumen present in the spores is so highly concentrated that the small quantity of water present is insufficient to bring about coagulation The spores also show an extraordinary power of resistance to low temperatures

The moulds differ to a marked degree in their sensibility to poisons *Botrytis*, for instance, is killed by a solution of formaldehyde four times weaker than that which destroys *Penicillium;* a fungus possessing extraordinary power of resistance to many poisons Thus it will withstand a solution of mercuric chloride sixteen times stronger than that which kills *Botrytis*. *Botrytis*, on the other hand, can withstand a solution of a toxic silver salt four times as strong as that which kills *Penicillium*.

The mould species described in the following pages, have been selected, partly on account of their morphological structure (representing types of development), and partly because of their occurrence in the fermentation industry, they belong to different sections of the system of "higher fungi"

The higher fungi are classified as *Oomycetes* (to which belongs the fungus causing the potato-disease, *Peronospora* or *Phytophthora*), *Zygomycetes* (to which *Mucor* belongs), *Ascomycetes* (including *Penicillium*), and *Basidiomycetes* (including the mushrooms). There are in addition a large number of fungi which are only known

to us in their conidial form, such as *Oidium*, *Dematium*, *Monilia*, and *Cladosporium*, which, it is assumed, represent a stage in the life-history of some of the higher fungi, as has indeed been definitely proved in certain cases These are known as "*fungi imperfecti*," a title which is not intended to be a reflection on the fungi, but solely to define our lack of knowledge respecting them

The general features of the **nutritive physiology** of fungi will now be dealt with The several species make very varied demands on the chemical constitution of the substratum Some are able to absorb certain of the chemical elements as such, but the majority assimilate them in the form of organic or inorganic compounds A large number of fungi are saprophytic, living on decaying substances, others adopt a parasitic mode of life, and derive their nourishment from the bodies of living organisms In dealing with nutrition, we distinguish between the process of assimilation, the conversion of the absorbed food substance into body substance, and that of dissimilation, the varied phenomena of decomposition and degradation due to the vital activity of the organism In this respect the most important of the vital functions is that of respiration The two processes are intimately related to each other During respiration, a large number of different nutritive substances are transformed and, in common with all other forms of life, the final products are carbon dioxide and water Amongst the products formed in presence of insufficient oxygen for complete combustion, mention may be made of oxalic acid, which is of remarkably wide occurrence in fungi In addition to the usual respiration of oxygen, a further kind of oxidation in absence of oxygen may take place, to which the term intramolecular respiration or respiration by dissociation has been given

The following facts may be of interest in regard to the elements of special value for the nutrition of fungi —*Potassium* appears to be essential to the growth of moulds, and particularly to the formation of their reproductive organs Within certain limits this element forms a useful food for yeast, and is believed to play a part in the nutrition of bacteria *Magnesium*, amongst the alkaline earths, is necessary for the generality of moulds, as well as for yeasts and many bacteria The formation of colouring matter in many bacteria has been shown to depend on the presence of magnesium in the nutrient fluid It may further be assumed that many fungi require *Calcium* in order to attain perfect development *Iron* appears to be required by moulds either as a food or as a stimulant, and, especially in the form of the sulphate, it reacts favourably on the propagation of yeasts We may assume that *Sulphur* is an essential element, since all albuminous substances contain sulphur; it is usually added to nutritive solutions in the form of sulphate Many fungi, however, seem to be capable of developing in nutritive solutions to which no addition of sulphur

has been made Sulphur plays a very important part in the metabolism of the sulphur bacteria *Phosphorus* is assimilated by fungi in a variety of compounds, and is, without doubt, a necessary constituent of their food The fluorescence exhibited by many bacteria depends upon its presence As is well known, *Nitrogen* is of the greatest importance as a foodstuff for fungi, and is assimilated by them in very varied forms It is assumed that some fungi are capable of absorbing and fixing nitrogen in its free state, others obtain their nitrogen in the form of inorganic compounds (ammonium salts, nitrites, nitrates), including species which can only absorb nitrogen from inorganic substances The most ubiquitous group of fungi, however, are dependent on organic compounds containing nitrogen, such as amides, peptones, albumoses, etc The first two groups must obviously have access to a supply of carbon, and this is often the case with the third group, for in many cases the nitrogen bound up in the organic substance can only attain its full value as a food in conjunction with other sources of carbon Both elements, however, usually occur together in the same chemical substance, and it is moreover impossible to draw a sharp distinction between any of the groups described. In regard to the relative food value of the substances themselves, it may be noted that, on the whole, the ammonium salts constitute a better source of nitrogen than the nitrates

When nitrates and nitrites are used, they must previously be reduced to ammonium salts, which directly serve to build up albuminoids Albuminoids and peptones have to go through a proteolysis before they can be utilised as N-sources. Concerning the application of amines, amides, and, more particularly, amino-acids (the best N-sources), authorities disagree, some of them holding that they are utilised direct, while others are of opinion that they must first be decomposed into ammonium salts.

The greatest diversity exists as to the sources from which the fungi may obtain their *carbon* Sugar, tartaric, acetic, oxalic and carbonic acids are amongst them In these, as in other cases, the value of the nutritive matter varies according to the other conditions of nourishment. The amount of aëration is an important consideration, since oxygen facilitates the absorption of certain substances, and is stimulating to some species, but acts restrictively on others Temperature, like aeration, may react in either direction. The nature of the nitrogenous compound, too, has a definite bearing on the availability of the source of carbon. In the case of yeasts, acetic acid forms an excellent carbon food for the Mycoderma. Citric and tartaric acids form specially good food for certain saccharomycetes, and, again, malic acid can be assimilated in considerable quantity by certain species Many bacteria also take up organic acids in the presence of nutritive salts. Glycerine and mannite are good sources of carbon for the moulds. Regarding

the nutritive value of **alcohol** for fungi, Pfeffer remarks (*Physiologie*, 1881) that dilute alcohol is a fairly good foodstuff for them. It appears from the experiments made by Duclaux, Linossier and Roux, Mazé and Perrier, Herzog and Polotzky, Schulz, Wehmer, Lindner, and others that those moulds and yeasts which need relatively large supplies of oxygen assimilate and oxidise alcohol of suitable concentration. The fungi, however, vary greatly in this respect. Some of them (such as *Mucor racemosus* and *M. javanicus*, according to Wehmer) prove to be unable to decompose alcohol, or to thrive on alcoholic mineral solutions, whereas other moulds (*e.g.*, *Penicillium glaucum*), and yeasts, develop readily in dilute alcoholic mineral solutions. It is, however, the **carbohydrates** which, as is well known, form the principal and most important foodstuffs. We shall return to these in the subsequent chapter on yeasts.

Speaking generally, one may assert that constructive activity predominates during the nutrition of green plants, whilst destructive activity predominates in the case of moulds; the enzymes which are of such general occurrence are the special destructive factors.

It may be remarked that certain substances, which are not necessarily incorporated by the fungi, are nevertheless of importance to them. The influence which those exert on the metabolism and growth of fungi suggests the term "**chemical stimuli.**" The presence of water and oxygen, for instance, is essential in bringing about the germination of spores, but it frequently happens that germination can only take place if certain substances are also present in solution. Klebs showed that the spores of *Aspergillus repens* will not germinate either in pure water or in inorganic nutrient solutions, or even on peptone, unless some inorganic salt, such as saltpetre, is added, but that, in a 0·5 per cent. solution of grape-sugar, germination does take place. Light also acts as a stimulant in certain cases, whilst in others it may have a retarding effect. It has been ascertained that minute doses of certain **poisons** have the effect of stimulating the growth of fungi, and of accelerating fermentative phenomena, an action which may, perhaps, be ascribed to physiological reaction on the part of the organisms concerned. Thus a small quantity of zinc sulphate (0.002 per cent.) added to a solution of sugar and inorganic salts has the effect of making the growth of *Aspergillus niger* twice as strong as under ordinary conditions. Copper sulphate has a corresponding action under certain conditions. In the case of yeasts, the addition of the merest trace of substances such as mercuric chloride (1 : 500,000), iodine, potassium iodide, chromic or salicylic acids has a very beneficial action on the fermentation. Lactic acid bacteria, grown in milk free from casein, are also stimulated by the addition of minute quantities of mercuric chloride.

The interesting observation has been made that products

formed by the metabolism of the fungi have a stimulating action on their growth, provided that certain conditions are observed, and the supply of nutrient substances is maintained.

These observations naturally lead to a consideration of the question of **external influences,** and their action on fungi in general.

An essential condition of growth of all organisms is the presence of **water** in such quantity as to create and maintain in the cells a condition of turgescence—*i.e.*, a hydrostatic pressure within the cell which keeps the protoplasmic lining in direct contact with the cell-wall. They require, however, only a minute quantity of water during the resting stage—*i.e.*, in the state of spores, and frequently these organs can withstand complete dehydration. It has already been mentioned that yeasts can be preserved in a dry state for a very long time, and spores of some of the moulds for several years. The concentration of the food, which is dependent on the amount of water present, plays an important part in the development of individual species.

It is well known that **temperature** is also an important factor. Growth is only possible above a certain minimum temperature, and it is accelerated with a rising temperature until at a certain optimum the organism attains its greatest activity. From this point on, if the temperature is still allowed to rise, the growth becomes less and less pronounced, and, finally, at a certain maximum temperature, ceases altogether. Different species exhibit very varied behaviour in regard to these three critical points, as is shown by the following examples:—

A number of yeast species have their minimum at 0.6° C., optimum at 28°-30° C., and maximum at 34°-40° C., whilst the corresponding figures are for:—

	Minimum	Optimum	Maximum
Penicillium glaucum,	1.5° C.	25° to 27° C.	31° to 36° C.
Bac. subtilis,	6° C.	approx. 30° C.	50° C.
Acetic bacteria,	8° C.	18° to 33° C.	30° to 36° C.

The limits of temperature may, moreover, vary in regard to the different organs of one and the same species (instances are found amongst the yeasts), and finally the results obtained in connection with any one particular species will vary according to the nature of the food supply.

In general, it may be remarked that organisms can withstand temperatures below the minimum without permanent injury. Thus, according to experiments by Schumacher, yeast cooled down to − 113° C. was not killed. Macfadyen subjected bacteria by gradual cooling to − 172°, and even to − 190° C., for a period of twenty hours, and mould spores to − 210° C. without killing them. On the other hand, a slight increase in temperature

above the maximum is often fatal. It is only spores of bacteria, particularly when dry, that are resistant to high temperatures. Many spores may, under these conditions, be heated for a short time to 140° C without injury

It is an interesting fact that some species of bacteria capable of thriving at a temperature of 0° C exist, and others, as stated, develop best at 50° C, or even 70° C

Light is of vital importance for the fungi, although not to the same degree as is the case with green plants The injurious effect which light has on the growth of bacteria, for instance, is now widely recognised, and it has been ascertained that for fungi in general both the nature of the nutritive medium and the temperature have a regulating effect on the action of light. From the physiological standpoint, the most active rays of light are the blue, violet, and ultra-violet rays

The natural purification of rivers is generally accepted to be due to the germicidal effect of light

Kny found that subdued light has no influence on yeast Lohmann, who used the intensive light of an arc lamp, but experimented at low temperatures, arrived at the same result He found that light has a retarding influence on the multiplication of yeast cells at 18° C and above, and that these were killed by prolonged exposure to direct sunlight, whilst diffused daylight delayed the process of budding

Many moulds can endure sunlight without injury, but intense illumination frequently restricts the longitudinal growth of the mycelial threads. Some species produce only mycelia in the dark, the reproductive organs requiring light for their development.

A number of experiments have been undertaken to test the effect of **electricity** on fermentation organisms Results have shown that the electric current has no influence, if care is taken to prevent the heat developed by the current from influencing the bacteria Whatever significance the electric current may be said to possess, such, for instance, as the preservation of fermented liquids, must be ascribed to the chemical effect of the current. By the treatment of water the number of germinable cells has been considerably reduced The action of **ozone** on water has already been referred to

It is a well-known fact that fungi are highly resistant to the influence of **pressure**. Bacteria, for instance, are found in the ocean at such a depth that they must be exposed to a pressure exceeding 100 atmospheres According to Melsens, yeast cells can withstand a pressure of 8,000 atmospheres. Chlopin and Tamman also found that bacteria, as well as yeast and moulds, could endure a pressure of as much as 3,000 kilos. per square centimetre. A rapid rise of pressure to this point, followed by instantaneous release, produced only a slightly injurious effect. The

fermentative activity was only retarded under long-continued pressure

With regard to the influence of **rest** and of **motion,** it may be observed that the organisms of fermentation thrive well both in and upon quiescent substrata Yeast also, as is well known, not only withstands active motion in its culture medium, but responds to it by a more active development The different species of bacteria respond in varying degrees to violent shaking Thus cholera bacilli cannot withstand shaking, but no effect is produced on typhus bacilli Buchner and Rapp showed that, whilst a slight motion has a beneficial effect on the fermentative activity of yeast, violent mechanical shaking has the effect of materially reducing this activity, the reduction being the more pronounced the poorer the fermenting medium

The action of **antiseptics** on fungi is intimately connected with the activity of the organism and with the temperature High temperatures augment the injurious effect of the reagent The degree of concentration of the substance is also of importance, for, as already mentioned, minute quantities exercise a stimulating effect In somewhat greater concentration the growth is retarded, whilst considerable quantities are fatal

In apparent contradiction with the above stands the remarkable fact that in certain cases more concentrated solutions of antiseptics have a less effect on bacteria than weak solutions, thus, a 3 5 per cent solution of cupric chloride killed the spores of the anthrax bacillus in a shorter time than that required by a solution of four times the strength

It is also known that different species exhibit a very varied resistance to the same substance, and to the same quantity of that substance Substances, moreover, which form suitable foods for some species are poisonous to others A great diversity of behaviour in regard to a particular poison is shown by one and the same fungus in its various stages of development, bacteria are more easily killed in the vegetative than in the spore condition Some fungi can withstand a certain amount of poison without their growth being restricted, but under such conditions, or even in the presence of a smaller quantity of antiseptic, they are unable to form reproductive organs The fungi possess the interesting power of adapting themselves to poisons when they are subjected to cumulative quantities An example has been mentioned in Chap. i. (hydrofluoric acid)

The **chemical constituents** of fungi are of a most varied nature. Water is the only one occurring almost always in relatively large quantities, the amount in bacteria representing about 80 per cent. ("Mother of Vinegar" forms an exception, and contains 98 per cent.) The yeast group contains from 40 to 80 per cent. The moulds and the vegetative parts of higher fungi contain from 80 to 90 per

cent, whilst in the resting and reproductive organs the percentage is much lower

In common with all organised bodies, the fundamental chemical constituents of fungi are the elements carbon, hydrogen, oxygen, and nitrogen. The relative amount of ash is found to vary enormously, the mycelia of *Aspergillus* and *Penicillium* contain about 6 per cent of ash The mineral substances which may be regarded as essential constituents of fungi are sulphur, phosphorus, chlorine, potassium, calcium, magnesium, iron, manganese, and sodium Sulphur and phosphorus are of special importance, as they form necessary components of the albuminoids Some idea of the importance of the phosphorus present may be gathered from the fact that P_2O_5 may often amount to half the total quantity of ash Chlorine, which apparently plays an important part in the nutrition of fungi, and which may be concerned in bringing about the dissociation of the nutrient fluids, probably occurs in all fungi. Amongst the metals, potassium occurs in the largest proportion, whilst calcium and magnesium are present in smaller amounts The same is true of iron and manganese, which, although occurring in comparatively small quantities, are nevertheless of the highest importance to the organism in the different phases of its life history Bertrand and Waterman showed that *Aspergillus niger*, for instance, does not form conidia in the absence of manganese (*Compt rend*, *Ac d sc* 154, 1912, *Acad Wet Amsterdam*, 21, 1913)

Cellulose, the carbohydrate which is of such general occurrence amongst the higher plants, is found also in the walls of fungoid cells, but the mucilaginous or viscous substances which often characterise the cell-walls of the former occur comparatively seldom Yeast mucilage is, however, a well-known example of this Foremost among the substances forming the contents of fungoid cells must be mentioned the **proteins or albuminoids,** which are in all probability the actual carriers of the vital phenomena They possess in addition a practical significance, yeast, for example, which is very rich in albuminoids, may be used for the preparation of nutritive media to take the place of meat-broth

The **nucleins** may be regarded as constituting a special class of albuminoids They have a most important biological significance, owing to the fact that the nuclei, as micro-chemical investigations have shown, are mostly composed of nuclein substance By application of pepsin the albuminoids in the cell are dissolved, but the nuclein compounds remain unchanged

Closely associated with the albuminoids, and, indeed, derived from them, are the **enzymes,** which doubtless occur in all forms of life They may in most cases be separated from the living cell, and can still produce their characteristic effects in aqueous solutions Each enzyme has the property of bringing about a

definite and particular form of chemical change, and the process is further characterised by the fact that a small amount of enzyme is able to decompose a relatively large quantity of organic substance

The result of the changes brought about by the action of the enzyme can be recorded in the form of a simple equation According to H Fischer, the enzymes may be classified as follows —

I. **Dissociating or splitting enzymes,** by means of which a complex substance is split into its component parts The action may be regarded as a form of hydrolysis, and is accompanied by the absorption of water The biological significance of these enzymes consists in enabling the organism to convert substances which would otherwise be useless into simpler and soluble substances capable of diffusion

The group includes —(1) Carbohydrate-splitting enzymes, such as invertase, which converts saccharose into equal molecules of dextrose and lævulose maltase, converting maltose into two molecules of dextrose, and lactase, which converts milk-sugar (lactose) into equal molecules of dextrose and galactose The sugars—saccharose, etc —can only be converted by fermentation into alcohol and carbonic acid after undergoing inversion.* The melibiase converts melibiose (a product of hydrolysis from raffinose = melitriose) into one molecule of galactose and one of dextrose. These enzymes are found in a large number of yeasts and moulds, and also in a few bacteria The diastases also belong to this group. They convert starch into dextrin and maltose, and occur in fungi, such as *Mucor* and *Aspergillus*. Cytase acts on cellulose, etc

(2) The glucoside-splitting enzymes, to which group emulsin belongs, an enzyme discovered by Liebig and Wohler in 1837. This enzyme changes amygdalin, a glucoside occurring in bitter almonds, into benzaldehyde, hydrocyanic acid, and glucose

(3) The fat-splitting enzymes (lipases), which decompose fats into glycerine and fatty acids

(4) The albumen-splitting or proteolytic enzymes (proteases), including pepsin, occurring in gastric juice, which convert albuminoids into albumoses and peptone; trypsin, which brings about further changes, and, more in our domain, the endotryptase of yeast

II The enzymes in this class have quite a different action from the foregoing They include the **oxydases,** the effect of which is to split up molecular oxygen, and thus render it active

Buchner and Meisenheimer showed that oxydase occurs in acetic acid bacteria, and also in yeast.

III **Reducing-enzymes** (catalases) These appear to occur in yeast and in certain bacteria

* As stated in the chapter on Yeast, it was observed by Willstatter that certain yeasts are able to ferment maltose or lactose direct

IV. A special group, the **fermentation enzymes, or zymases**, the chemical action of which is entirely different. They bring about true fermentation, which has been defined by H. Fischer as an intro-molecular readjustment of oxygen, accompanied both by oxidation and reduction of the several carbon atoms, and an increase in the compounds of carbon and oxygen; the usual result of the process being the breaking up of a single molecule into several. In addition to the true zymase (or alcoholase), this group includes the enzyme of lactic acid fermentation. A further description of these enzymes will be found in the chapter on alcoholic yeasts.

The activity of enzymes is influenced to a very considerable degree by temperature. The optimum for zymase lies at about 30°, for pepsin about 40°, and for the proteolytic enzymes (*e.g.*, barley malt), about 60° C.

The action of dilute acids and alkalies is also very varied. Thus, minute quantities of free hydrochloric acid ($\frac{1}{3000}$ of normal acid) greatly increase the activity of invertase.

The action of enzymes is to a great extent analogous to that of warm acids (hydrolysis).

The formation of the enzyme by the fungus is dependent to some extent on nutrition, as already stated in the chapter on bacteria. *Aspergillus glaucus*, for instance, as Duclaux showed, when cultivated on a solution of calcium lactate and nutrient salts, secretes diastase, but no invertase. On the other hand, on a solution of cane-sugar and nutrient salts, invertase is produced, but no diastase nor any other enzyme that it is capable of producing. Only by cultivating the fungus on milk is it capable of producing clotting enzymes and casease. Similarly, it was discovered by Went that a species of *Monilia*, which normally contains a number of enzymes, could only form certain of them, such as trypsin and the clotting enzyme, when substances capable of being split up were present. Fermi proved that several kinds of bacteria, when cultivated on media free from albumen, formed no albumen-splitting enzymes, and that *Bacillus subtilis* only produced diastase when fed with some form of peptone.

In fungi, moreover, a number of poisonous substances occur—ptomaines, toxins, etc. These are not to be regarded as produced exclusively by the higher fungi (toadstools, etc.), but occur also in the lower forms, such as rusts and smuts, which have caused symptoms of poisoning. Reference may also be made to the extremely poisonous nature of *Aspergillus fumigatus* and *A. flavescens*.

The carbohydrates which have been shown to occur in fungi include glucose and lævulose; mannite is very widely distributed, and glycogen, to which we shall return in the chapter on alcohol-producing yeasts, generally occurs as reserve substance.

Fats and free fatty acids are found as reserve substances, and

as secretions in many fungi *Penicillium* and *Aspergillus*, for example, have been shown to contain from 4 to 5 per cent. of these substances Yeast stores up fat along with glycogen, the former constituting from 2 to 5 per cent of its dry weight or even more under specific conditions Amongst organic acids, oxalic acid is widely distributed

A whole series of colouring matters are found in the cells of these plants The colouring matter present in so many of the bacteria is specially interesting, and has been shown to play an important part in their vital economy The fatty colouring matters or dye-stuffs combined with fatty acids (lipochromes) are of frequent occurrence Allusion may also be made to tannins, resins, and ethereal oils.

1 Botrytis cinerea (*Sclerotinia Fuckeliana*).

Botrytis cinerea forms small greyish-yellow patches on moist, living or decaying vegetable matter, and may also occur on wort. From the greyish-brown mycelium the conidiophores are thrown up; these are perpendicular, articulated filaments, generally arranged in tufts They grow up to a height of 1 mm, after which the apical cell throws out near its point and almost at right angles, from two to six small branches The lower branches are the longer; at a short distance behind their apices, these again give rise to one or more short side branches The topmost branches are almost as wide as they are long Thus a system of branches is formed shaped like a cluster of blossom or a bunch of grapes. When longitudinal growth is at an end, the interior of the branches is separated from the main stem by the formation of a transverse wall close to the latter. At the same time the ends of the branches and of the main stem swell, and on the upper half of each swelling several small papillæ appear close together, these quickly increase to oval blisters, filled with protoplasm, and grow narrow and stalk-like at their base When these conidia are completely developed, the walls of the branches carrying them shrivel up, and the conidia are consequently brought so closely together that they form a loose, irregular accretion, which readily falls off. If these clusters are placed in water, the conidia detach themselves from their stalks, and the envelopes of the branches, devoid of protoplasm, shrivel up or are only to be found in traces; their former place of attachment to the main filament appears only as a slightly raised scar. The member immediately below can now displace the shrivelled apex, grow upwards, and form a new cluster, this may be repeated several times, whereby the conidiophores attain a considerable length According to the observations of Rindfleisch, the formation of conidia takes place only during the night. Klein and Lindner found that, in daylight, the more strongly refracted portion of the

spectrum, the blue-violet rays, hindered the formation of conidia, whilst it is encouraged by the red-yellow rays. Under the constant influence of red-yellow light, and in total darkness, the production of conidia goes on both day and night. Other workers arrived at different results. Thus, Moreau used a Nernst lamp placed at a distance of about one and a half metres from the slit of the spectro-

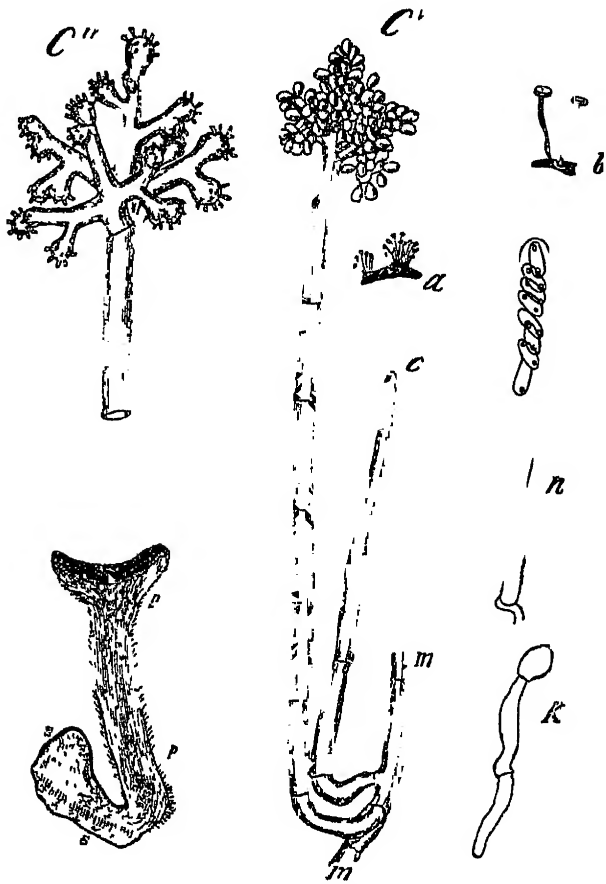


Fig. 32.—*Botrytis cinerea* (after de Bary).—*a*, *b* (natural size), Sclerotia, from which at *a* the conidiophores, at *b* the apothecia (fruits with asci), are thrown out; *c*, *C*, conidiophores (*C'*, with conidia just ripe), springing from the mycelium filament *m*; *C''*, end of a conidiophore with the earliest formation of conidia from the ends of the branches; *k*, germinating conidium (× 300); *p*, *s* (slightly magnified), section through a sclerotium *s*, from which a very small apothecium (*p*, *p*) is thrown up; *n*, single ascus, with eight ripe spores (× 300).

scope, and a carrot culture of the fungus was exposed for some days to the action of the spectrum of this source of light. Under these conditions conidia and a luxuriant mycelium were only formed by the action of the violet and blue rays, whereas the red, orange,

yellow, and green rays retarded the formation of mycelium and prevented the development of spores Under certain nutritive conditions the conidia and ascospores develop short threads, from which small, bright, round conidia are separated, either directly or on bottle-shaped basidia These conidia are not capable of germination If the mycelium has been cultivated for some time on a solid substratum which it is incapable of penetrating, short branches are formed, which by further and repeated branching have the appearance of closely arranged tufts or tasselled knots. They lie in close contact with the substratum, and form the characteristic organs of attachment.

Under certain conditions this mould can assume a peculiar dormant state, the **sclerotium** (*skleros* = hard). The hyphal threads branch out freely, and the branches intertwine forming a compact body of varying shape, circular or fusiform, and of varying size, the extreme ends of the filaments are brown or black, and the ripe, solid sclerotium thus consists of an outer black rind and an inner colourless tissue These bodies, which were described by de Bary under the name of *Sclerotinia Fuckeliana*, occur as small black bodies on the herbaceous parts of many plants, where they live as parasites or saprophytæ They are capable, after a long period of rest—lasting at least a year—of forming a new growth. If the sclerotium is brought into a moist place soon after it comes to maturity, the inner colourless branches break through the black outer rind and develop into conidiophores If, however, the sclerotium is only brought into a moist place after it has been at rest for some time, a large tuft of filaments develops from the inner tissue, and these shoot up perpendicularly, and finally spread out to a flat, plate-shaped disc, the ends of the filaments are arranged in parallel rows on the free upper surface of the disc; some of them remain thin, others swell up to club-shaped asci, and each of these asci forms in its interior eight oval spores. The mould has now entered upon the stage in which the formation of **apothecia** takes place The spores germinate when they are set free, and the germ tubes grow into conidiophores.

In rainy seasons, when *Botrytis* attacks the unripe grapes, the mycelium, penetrating through the pulp, destroys the small amount of sugar in the grapes, and, as it kills the cells, a fresh migration of sugar from the leaves is checked or rendered impossible. Such grapes act injuriously upon the quality of the wine. As the mycelium penetrates into the stalks also, causing them to die off, the very young grapes on such a cluster do not usually develop, but wither away. In red grapes it attacks the colouring matter and converts it into a compound insoluble in the fermenting liquid. It has also been observed that it is apt to cause viscosity in wine when it occurs in large quantity in the must.

In years of good vintage the fungus does not usually appear

until just before the grapes are gathered, and then gives rise to a different set of conditions. According to Muller-Thurgau's investigations the mycelium spreads principally through the skin of the grape, which becomes brown, leathery, and permeable to moisture Thus in dry weather part of the water evaporates, the juice becomes more concentrated, and the grapes wither The fungus does not penetrate far into the interior of the grape, but its growth affects both the acid and the saccharine constituents of the juice, and the must obtained from such grapes appears relatively richer in sugar and poorer in acids than usual Under favourable climatic conditions, especially in a dry atmosphere, white grapes which have been attacked by this "Edelfaule" can thus produce a must weak in acid, and hence yields a wine of finer quality and of special bouquet, due to some extent to the action of the fungus This is particularly the case with varieties of vine which yield hard grapes with a high sugar and acid content—*e.g*, the Riesling vine A certain element of danger may, however, lurk in these attacks on the grapes, partly because *Botrytis* absorbs part of their albuminoids, rendering the must less nutritious for the yeast, so that the latter develops more slowly, and partly, as shown by de Bary and, later, by Behrens and Muller-Thurgau, because the fungus secretes a poison which prevents the development of the yeast Wines of this description, therefore, mature slowly, and are exposed to the attacks of foreign organisms The fungus is invariably harmful to red and blue grapes, even if they are attacked when ripe

Some species of *Botrytis* contain an enzyme which destroys cellulose

It may be mentioned in conclusion that the leaves and stems of tobacco plants are subject to the attack of certain species of *Botrytis*, which bring about decay.

2 Penicillium.

A large number of species belonging to the genus *Penicillium* have been described in recent years Many of them, however, seem to be mere varieties of far less numerous types A considerable number of the species have much the same shape as that described for *P glaucum* The most suitable culture medium is meat-peptone-gelatine with sugar and glycerine, or neutralised Raulin's solution with gelatine. Woltje, as a specially adapted nutrient solution, recommends 1 g. of asparagine, 0·5 g. K_2HPO_4, 0 25 g. $MgSO_4$, and 7·5 g cane-sugar in 100 c c of water A characteristic feature of these fungi, as of *Aspergillus*, is that the colour and general appearance of the growths vary according to the culture medium.

A classification of the different species has been made by Biourge, Lindau, Wehmer, Westling, Sopp, and others.

A species which is widely distributed in the fermentation industries, especially in green malt, is *Penicillium glaucum*. It forms a felt-like mass on the substratum, at first white, then greenish or bluish-grey, and it spreads with great rapidity. The mycelium consists of transparent branched and divided filaments, which, when immersed in liquids, are liable to swell somewhat irregularly. From these filaments the conidiophores rise perpendicularly. They consist of elongated cylindrical cells, the terminal cell of which is soon arrested in its growth, and forms a thorn-like point; the cell immediately below throws out one or more opposite branches, rising up close to the terminal cell, each consisting of a single-pointed cell. In more vigorous individuals the branches may again ramify, or similar branches may also spring from the next cells, and these also ramify and become pointed as described above. In this tuft of branches each pointed cell (**sterigma**) breaks up into a series of spherical conidia, and finally the tuft carries a large number of conidia, arranged in series, which, when ripe, are readily scattered. These round, smooth conidia give to the patches of mould their greyish-blue colour; when they fall upon moist surfaces, they are able to germinate at once. According to Cramer, they are very resistant to higher temperatures. Under certain conditions it may happen that several conidiophores unite so as to form a column surmounted by a round headed group of sterigmas and conidia (*Coremium*).

In culture experiments with this fungus, Brefeld made the interesting observation that *Penicillium* may occur under certain conditions with an entirely different form of growth. He enclosed cultures of this mould on slices of coarse, non-acidified bread, between glass plates, and allowed the culture to develop whilst excluding air as far as possible. Pairs of short, thick branches grow out from the mycelium, which coil round each other; one part of this spiral throws out short, thick tubes, whilst the hyphal thread carrying the spiral develops numerous fine branches, which envelop it and form a covering consisting of a dense inner and a felted outer layer; the inner cells gradually turn yellow, and the loose outer cells are cast off. In this small yellow ball a formation of swollen cells gradually takes place by the continued branching of the spirals, and in each of these new cells eight large and lenticular spores are produced, which have a circular furrow on the margin, and three or four slight ridges on the outer membrane (exosporium). After the collapse and absorption of all the remaining contents, the spores are set free and the small yellow ball is then filled with the spore dust. The entire development requires six to eight weeks. The ascocarps may be preserved in a dry state for several years without losing their power of germination. When the spores are sown, the exosporium bursts open like a shell at the circular furrow, and the endosporium swells, emerges

and elongates itself to a germ tube, which quickly develops conidiophores. As already noted in the introduction to this chapter, these fructification corpuscles were found by Bezssonoff to form in cultures on highly concentrated media containing cane-sugar and nutritive salts.

This fungus often causes dangerous diseases in wine It develops freely in casks which have not been carefully cleaned, penetrating into the wood, and produces decomposition products of

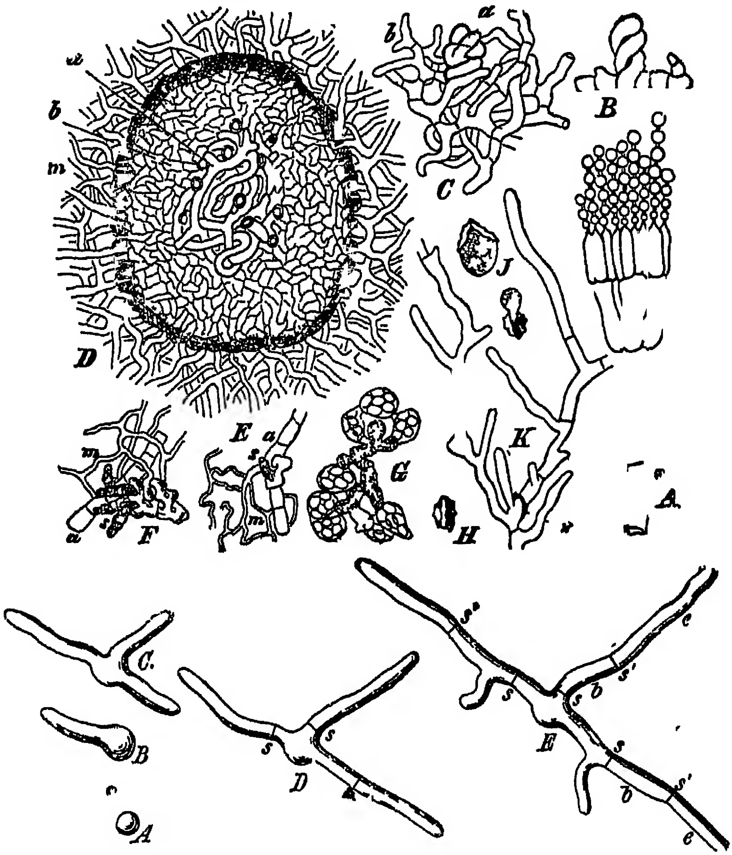


FIG 33.—*Penicillium glaucum* (after Brefeld and Zopf) —*A*, Conidiophore, *B*, organs of generation, *C*, first development of the sclerotium (*a*, ascus-forming hyphæ, *b*, sterile filaments), *D*, very young sclerotium in section (*a*, ascus-forming hyphæ. *b*, sterile portion of the sclerotium, *m*, mycelium); *E* and *F*, ascus-forming hyphæ, (*a*) with young asci (*s*) and sterile mycelia (*m*) from a more developed sclerotium; *G*, group of asci with spores, *H*, spore, *J*, germinating spores, *K*, young mycelium (with spore at *x*) *A*-*E* (below), germination of a conidium, after Zopf (more highly magnified), *A*, conidium before germination, *B*, it has thrown out a germ tube, *C*, three germ tubes have been formed, *D*, each germ tube shows a transverse septum towards the spore (*s*), *E*, each germ tube has been divided by another septum (*s'*) into a terminal cell (*e*) and an inner cell (*b*)

disagreeable smell and taste, which subsequently diffuse into the wine In moist seasons it forms a dense growth on grapes, attacks the sugar contents of the fruit, and brings about a peculiar decomposition The mycelium seems to penetrate not only into bruised but also into sound grapes They gradually acquire a yellowish-brown or greenish-yellow colour, and the fungus produces those well-known decomposition products which cause the mouldy taste in wine The conidia of this fungus may exist for a long time in must or in wine, upon which the germinating mycelium exerts a deleterious effect

According to Wortmann, *Penicillium* has a particularly harmful effect on bottled wines It penetrates the corks, thus giving rise to the corked flavour, and it may even grow right through the cork and develop in the wine, attacking some of its constituents and rendering it turbid

The following species are of special importance for industrial purposes —

P. Roqueforti, which imparts to Roquefort cheese its particular taste It bears some external resemblance to *P. glaucum*, from which it distinguishes itself by its conidia being twice as large (4 to 5 μ against 2·5 μ). The growth is dark-green, later dirty-brown and whitish-yellow below The conidia very quickly (under good conditions in 30 to 40 hours) form a new mycelium with conidia, and long retain their germinating power The growth is almost odourless. Whilst growing it decomposes free lactic acid, converts milk-fat into fatty acids, and peptonises casein, with formation of ammonia *P Camembert* (*P album, candidum*), which appears while Camembert cheese is ripening, and also in Brie cheese, comprises different strains forming a white covering, afterwards turning greyish or grey-green The ripe conidia are spherical, smooth, 4 to 5·5 μ diameter They form only on free surfaces. Dworak found these species possess much the same chemical properties as *P. Roqueforti*.

P. luteum has a yellow mycelium, changing green or light brown when covered with conidia. The conidia are small and elliptical, and the sterigmata exceptionally long. This species forms yellowish-brown ascocarps, the ascospores are provided with prominent transverse stripes. It usually occurs on fruits, causing them to decay, but it may also occur on a variety of other substances. *P. italicum* seems only to affect lemons and similar exotic fruits, causing their decay. It forms a greenish-blue covering, and has ellipsoidal conidia somewhat larger in size than those of *P. luteum*. *P. olivaceum*, occurring on exotic fruits, and less frequently on others, has a distinct greenish-brown colour and large ellipsoidal conidia.

Most species of *Penicillium*, and especially *P glaucum*, contain a number of different enzymes, one of which, a proteolytic enzyme,

is present in all species hitherto examined. *P. glaucum* also possesses a diastase which, according to Laborde, converts starch into dextrin and dextrose In addition, they contain invertase, maltase, inulase, a clotting enzyme, a casein-splitting enzyme, etc, and a poisonous substance which has not been identified

The genus *Citromyces* demands special notice The thin-walled conidiophores are not usually ramified, they are without any swelling at the end or with a vescicular one, and have a terminal cluster of sterigmata The conidia are small and mostly globular. This fungus forms a covering of many colours on sour fruit; also develops readily on concentrated solutions of organic acids (*e g*, tartaric acid 25 per cent or a saturated solution of oxalic acid). A satisfactory medium is a decoction of white beans A number of species have been described by Wehmer, Mazé, Sopp, Biourge, and others A particular interest attaches to the fact that several of these species (like *Aspergillus niger*) are employed for industrial purposes, such as the manufacture of citric acid, which is generated by them in the course of their growth, and can be isolated if neutralised by carbonate of lime as fast as it forms. Citric acid is not formed direct from sugar, but through the medium of more complex compounds, the formation is closely connected with the metabolic changes taking place in the cells, and does not begin until a considerable part of the nitrogenous matter has disappeared. The fermentation is an oxidation process, it goes on most actively with sufficient access of oxygen at a moderate temperature. The details of the chemical processes involved have not yet been cleared up. In competition with other micro-organisms the citromyces species are soon suppressed, even slight contamination by yeast is apt to interfere seriously with citric fermentation.

3. Aspergillus.

The most commonly occurring species is *Aspergillus glaucus*, first fully described by de Bary. It forms a fine, greyish or greyish-green felt on various materials, and grows with great luxuriance on green malt.

The mycelium consists, as in the case of *Penicillium*, of fine transparent and branched threads, provided with transverse septa. Some of the hyphal threads grow up perpendicularly, are thicker than the rest, and are rarely branched or divided by septa. The upper ends swell to spherical, flask-shaped heads, and these throw out from their entire upper portion radially divergent papillæ of an oblong form, the sterigmata then develop at their apex small round protuberances, which are attached to the sterigmata by much constricted bases, and after some time break off to form independent cells (conidia). Below the base of the first conidium a second begins to form from the crown of the sterigma, and pushes

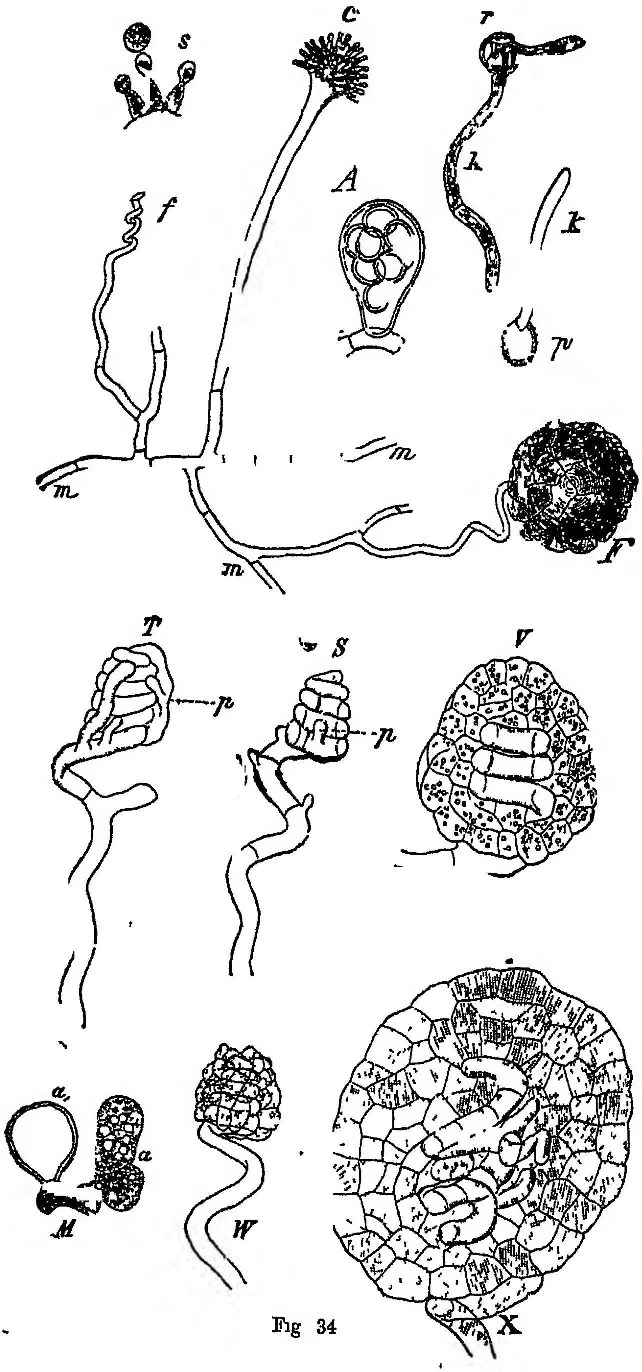


Fig 34

Fig. 34—*Eurotium aspergillus glaucus* (de Bary)—*m*, *m*, hyphal thread, carrying a conidiophore *c* (from which the conidia have fallen), a perithecium *F*, and the first rudiments of an ascogonium, *f* (× 190), *s*, three sterigmata from the crown of a conidiophore, showing the conidia-constrictions; *p*, germinating conidium (× 250-300); *A*, Ascus; *r*, germinating ascospore; *k*, germ tubes; *S*, spiral ascogonium; at *p* the commencement of the growth of one of the enveloping hyphæ; *T*, older stage; *W*, ascogonium, already surrounded by the envelope; *V*, longitudinal section of an older stage; in the centre the ascogonium, surrounded by the envelope, consisting of several layers, *X*, longitudinal section of a later stage of development; the ascogonium is enveloped in a sheath of many layers; it has loosened its convolutions, and is beginning to throw out the ascus-forming branches; *M*, portion of an older ascus-bearing branch; *a*, a young ascus; a_1, an older ascus after bursting.

the first upwards, a third then forms, and so on Each sterigma thus carries a chain of conidia, the youngest of which lies closest to it This occurs simultaneously over the whole surace of the enlarged end of the conidiophore, which is thus inally covered with a thick head of radially-arranged chains of conidia These masses form the greyish-green dust which covers he mycelium

Finally, the conidia separate, and then have a warty appearance on their surface These small bodies can germinate as soon as they are detached, and at once develop a new mould, upon his fact depends the rapidity with which the plant spreads Under avourable conditions, the fungus develops *perithecia* They appear at first as tender branches, which, at the termination of their ongitudinal growth, begin to twine their free ends in the form of spiral of four to six turns, the threads of the spiral gradually pproach nearer together, until finally they come into contact, so hat the whole end of the filament takes the form of a helix (the **scogonium**) Two or more small branches which cling closely o the spiral then grow from the lowest turn of the helix. One of hese quickly outstrips the others in growth, its upper extremity eaches the uppermost turn of the helix, and fuses with it. The other ranch or branches likewise grow upwards along the spiral, shoot ut into new branches and gradually become so interlaced that the piral is finally surrounded by an unbroken envelope. The ranches divide slowly into septa perpendicular to the surface, and he envelope consequently consists of short, angular cells, in which ew septa appear parallel to the surface, so that the envelope thickens nd is composed of many layers The small sphere now formed is bout 0·25 mm. in diameter; the outermost layer is first yellow nd then brown, the inner layers remain soft, and are finally issolved After a time the spiral extends and throws out on all des branched filaments, which dislodge the inner layers of the nvelope. These branches finally take the form of an ascus, eight pores being formed in each After the breaking up of the asci the pores lie loose in the interior of the perithecium, and are liberated y the rupture of its fragile wall The spores are bi-convex, and arry a longitudinal furrow, they possess an opaque outer memrane and an inner one, which on germination bursts the outer embrane, forming two valves This species thrives best at a mperature of about 25° C. The enzymes that are associated ith *Aspergillus glaucus* are, *inter alia*, diastase, invertase, maltase, id a proteolytic enzyme

Another well-defined form is *A. flavus*, with a yellowish-green ycelium occurring frequently on bread, and also on dry excreent. Its optimum temperature is 37° C, and it is believed be pathogenic, its presence having been detected in the human r. Its conidia are usually smooth.

A. fumigatus occurs on very different substrata, and is also pathogenic both to man and beast It forms a greyish or greyish-green vegetation, and has an optimum temperature of 40° C It produces minute conidiophores with small spherical conidia and brown perithecia. It contains the same enzymes as those occurring in *A glaucus* It plays a part in the spontaneous ignition of dry vegetation, and may, according to Cohn, cause a rapid rise of temperature in green malt

A particularly interesting form of *Aspergillus* is *A. (Sterigmatocystis) niger*, which produces branched sterigmata It is of very general occurrence and forms blackish-brown patches of conidiophores, together with spherical, smooth, or warty conidia. It also produces yellowish sclerotia (without spores) Its optimum is about 35° C * It grows well in an extract of oak galls, and in a tannic acid solution. It is of technical importance, as it is used in the preparation of gallic acid from tannin. According to Fernbach and Pottevin, a special enzyme secreted by the fungus is active in this process It is produced only on substances containing tannic acid, and has its optimum at 67° C.

This species further possesses diastase, invertase, maltase, proteolytic enzymes, inulase (according to Kiesel, the formation of the last enzyme is promoted by very weak acids), and emulsin (which according to Javillier consists of two enzymes and has its optimum about 56°-60° C) Like many other fungi, it forms **oxalic acid,** which occurs as an intermediary product before the complete combustion of the sugar to carbon dioxide, as shown by Duclaux, who developed it on Raulin's solution This acid was found by Wehmer to be sometimes absent during the growth of the fungus, whereas it was invariably formed in the process of metabolism when KNO_3 is the source of nitrogen, the potash set free being neutralised by the acid , whilst with NH_4Cl as the source of nitrogen no oxalic acid can be found.

According to Elfving, the formation of oxalic acid on pure sugar solutions is impeded by certain ammonium salts as sources of nitrogen, such as ammonium chloride, sulphate, and nitrate On the contrary, the amount of oxalic acid increases if certain calcium salts—$Ca_3(PO_4)^2$ or $CaCO_3$—are used As oxalic acid is an oxidation product, the presence of free oxygen is necessary ; at low temperatures, say 19° to 20° C , a greater accumulation takes place than at higher temperatures The oxalic acid is probably not formed direct from the sugar, but only through the demolition of more complex compounds. It is of particular importance to note that **citric acid** also occurs in these fermentations , this acid is, however, consumed sooner than the oxalic acid, because it has a greater nutritive value for the fungus According to thorough

* As noted above, Bertrand and Watermann found that no conidia are formed when the nutrient liquid does not contain manganese

investigations by Elfving, who developed the mycelium in a suitable nutrient solution,* which after careful washing was replaced by a sugar solution (at 21° C), the fungus on addition of $Ca(NO_3)^2$ or $CaCl_2$ produces citric acid, the formation of oxalic acid being arrested. The races appear to differ in the ratio of the two acids produced This may prove the existence of physiological races within this otherwise uniform species, yet, according to Elfving, it is possible, in a fungus that produces oxalic acid, to entirely suppress the latter by the cumulative action of citric acid On the other hand, Elfving found that there exist races which tend to generate citric under circumstances where otherwise oxalic acid is produced

In the preparation of the strongly fermented Japanese rice wine (saké), *Aspergillus Oryzæ* is systematically employed This fungus forms yellowish-green patches, and grows on the most varied media The conidiophores terminate in spherical or club-shaped swellings, and the sterigmata radiate either from the upper portion, or from the entire surface of the swollen end of the conidiophore The conidia are large and yellowish-green, they are either oval or spherical, and may be either smooth or covered with fine warts According to Wehmer, they can maintain their vitality for many years, a fact which has been substantiated by observations made in the author's laboratory The existence of a yeast stage of this fungus, due to the budding of the conidia, has also been confirmed by direct observation of the author. Zikes observed on the mycelium a formation of gemmæ with agglomeration of budding cells, when grown in a nutrient liquid containing 100 c c water, 7 5 g of saccharose, 1 g. ammonium sulphate, 0 5 g K_2HPO_4 and 0 25 g $MgSO_4$ We have seen that Bezssonoff, who cultivated the fungus in concentrated sugar solutions, observed a development of sexual hyphæ with distinct ascogones at 30° C, and afterwards at room temperature A further development into ripe perithesia was observed by Zikes in a solution of 1 g asparagine, 0·5 g K_2HPO_4, 0 25 g $MgSO_4$, and 7·5 g of saccharose in 100 parts of water, with or without gelatine. The perithecia develop as yellow globules containing spherical asci with eight smooth spherical or ellipsoidal spores

To prepare saké, rice grains, freed from their hulls, are steamed, but the aggregation and gelatinisation of the grains must be avoided. In order to prepare a malt serviceable for brewing from these grains, which are unable to germinate or to exercise the usual diastatic activity, the mass of grain is mixed with other rice grains, which are coated over with the mycelium and conidiophores of *Aspergillus Oryzæ*, or else the yellowish-green spores ("Tane-Koji")

* 0·5 per cent of ammonium chloride or nitrate, 0 25 per cent monopotassium phosphate, 0 12 per cent cryst magnesium sulphate, and 5 per cent dextrose or saccharose, 3 days at 35° C.

of the fungus are mixed with the steamed rice grains In moist and warm air, after the lapse of about three days, a white velvety mycelium is developed on the rice, which imparts to the mass an agreeable odour resembling apples or pineapples Before the fructification of the fungus takes place, a fresh quantity of steamed rice is introduced, and this also is gradually coated with mycelium, the process is repeated several times In the "koji" mass thus produced, a part of the starch has been hydrolysed, and some of the albuminoids have been rendered soluble The koji mass is mashed in the cold, 21 parts of koji being mixed with 68 parts of steamed rice and 72 of water This pasty mass ("Moto") is allowed to remain at about 20° C, after some days it clarifies The conversion of starch and dextrin into sugars progresses, and at the same time a spontaneous and very violent alcoholic and lactic fermentation sets in In this fermentation there occurs a *Saccharomyces* which is able to produce a very high percentage of alcohol The mass is now warmed to about 30° C After two to three weeks the primary fermentation is finished. The product, after being filtered, is subjected to a secondary fermentation, and the liquid is then clear and yellow, like sherry, containing 13 to 14 per cent of alcohol It is usually pasteurised at 50°-70° C. in iron vessels

According to Kellner, *Aspergillus Oryzæ* also plays an important part in the preparation of Japanese Shoyu or Soja. Saito and Kita made a thorough biological study of the manufacturing process, which is carried out on the following lines —The raw materials used are soy beans boiled under pressure, and parched, crushed wheat grains From a mash thus prepared "koji" is made by inoculation of *Aspergillus Oryzæ*, or merely by spontaneous development of the fungi contained in the vessels With access of air, the temperature rises gradually to 38°-40° C, with abundant development of mycelia and conidia The fermentation is complete in three days, when the koji is mixed with salt water in a vessel of about 40 hectolitre capacity, where the development of mould and various species of yeast and bacteria is carried on. Alcohol and lactic acid are gradually produced, together with the products of the action of proteolytic bacteria The characteristic aroma develops in the course of a year After dilution with water, the mash is extracted and filtered, the filtrate being pasteurised in iron boilers and clarified by being allowed to stand or by refiltering According to Kellner, *Aspergillus Oryzæ* is also of importance in the preparation of the Japanese bean mash (Miso).

Korschelt found that the hyphæ of this species secrete a diastase, which, like malt diastase, converts starch into dextrin and maltose, an observation confirmed by Atkinson, and subsequently by Cohn and Büsgen.

Takamine prepared this diastase for industrial use, to supply

the place of malt For this purpose he sows a pure culture of the fungus upon wheat bran previously sterilised by steaming and cooling to 40° C The material is spread in thin layers (*e.g*, in tanks with a double wire mesh bottom) The conidia are developed at 30°-35° C in air saturated with water vapour, the temperature rising for a day and night, and then being allowed to drop slowly, the mass acquires its maximum of diastatic power when the fungus has grown through the undermost layer of the nutrient medium, and the mass is then dried in a current of dry air, until it contains 10 to 15 per cent of moisture The full diastatic effect was obtained from 4 per cent. of this very durable "taka-koji" in 15 to 20 minutes By precipitation with alcohol the diastase ("taka") can be obtained as a white hygroscopical powder, containing invertase, maltase, cytase, oxydase, peptase, erepsine, tryptase, lipase, and a starch-diastase The enzyme action is increased by addition of sulphuric acid in the proportion of 1 2,000 According to Bertrand, the taka invertase distinguishes itself from that of alcoholic yeast and *Aspergillus niger* by the fact that it is most active in neutral or slightly alkaline liquids

Atkinson found an enzyme in koji which is soluble in water, inverts cane-sugar, and converts maltose, dextrin, and starch-paste into dextrose The researches of Kellner, Mori, Nagaoka, and Okumura have likewise shown that the koji mass possesses a strongly invertive enzyme, which converts cane-sugar into dextrose and lævulose, maltose into dextrose, and starch into dextrin, maltose, and dextrose. The various micro-organisms which occur in the koji mass doubtless contain these different enzymes. Saito observed a peculiar kind of acid formation due to this ferment

In Java, the *Aspergillus Wentii*, described by Wehmer, is used for the preparation of Chinese soja, and the "Tao-Tjiung" (bean mash). It occurs spontaneously on soja beans. It forms a snow-white mycelium, coloured brown at a later stage by the globular conidia, which exhibit a fine warty structure, the sterigmata are not ramified Bezssonoff, cultivating this species in concentrated sugar solutions, observed an incipient formation of sporangia as in the case of *A. Oryzæ* According to Prinsen Geerligs, who described the technical application of the fungus, it not only possesses a peptonising and diastatic ferment, but is also capable of partially dissolving the cell-walls of the soja bean When the boiled soja beans have been sufficiently acted on by the fungus, they are mixed with a concentrated salt solution, and the mixture boiled with sugar and various aromatic herbs The process is, therefore, not one of fermentation.

Saito has investigated the preparation of the Japanese yam brandy, involving the use of a special kind of *Aspergillus* (*A. Batatæ*), by means of which the starch of the yam tubers is

converted into sugar This species first forms yellowish-green, changing to dark brown patches, consisting of brown, spherical and finely grained conidia. It possesses the same enzymes as other species of *Aspergillus* The alcoholic fermentation is brought about by a special form of yeast, *S Batatæ*

4 Mucor.

The genus *Mucor* belongs to the most interesting group of moulds with which we have to deal, since it embraces species with marked fermentative activity and great power of hydrolysing starch They occur as grey or brown, felt-like masses, often attaining considerable thickness—occasionally measuring several inches—in which small yellow, brown or black globules may be distinguished by the naked eye Only the more commonly occurring species are described.

Mucor Mucedo, one of the most beautiful mould growths, and one which occurs very generally on the excreta of herbivorous animals and especially on horse excreta, has a transparent white mycelium, which develops numerous and delicate ramifications both above and below the surface of the substratum. In its earliest stages of development, and until the sporangia begin to form, it is without transverse septa, and, therefore, unicellular. Single vigorous branches, the sporangiophores, rise from the mycelium; the points of these branches, which contain a reddish-yellow fatty colouring matter, swell greatly, the protoplasm withdraws from the stalk into the enlarged heads, and below the swelling a transverse septum is finally formed, whereby the sporangium is cut off from the sporangiophore. The transverse wall arches upwards, and forms a short column (columella) in the interior of the spherical head, whereby an inner space of peculiar form results. The protoplasm of this space breaks up into a number of small fractions, which are gradually surrounded by a membrane and rounded off; these are the spores At the same time the sporangium is coated on its outer surface with small needle-shaped crystals of calcium oxalate. As soon as the ripe, greyish-brown sporangium takes up moisture, the wall dissolves, and the spores with their yellowish contents are scattered on all sides along with the swelling contents of the sporangium The columella, which projected upwards in the sporangium, still remains at the end of the sporangiophore, this is now surrounded at its base by a collar, the remains of the outer wall of the sporangium When the refractive spores fall on a favourable substratum, they swell very considerably, and send out one or two germ tubes, which quickly develop into a vigorous mycelium. The optimum for growth lies between 20° and 25° C

In addition to this mode of reproduction, *Mucor Mucedo* and the other species possess a sexual method of reproduction, which

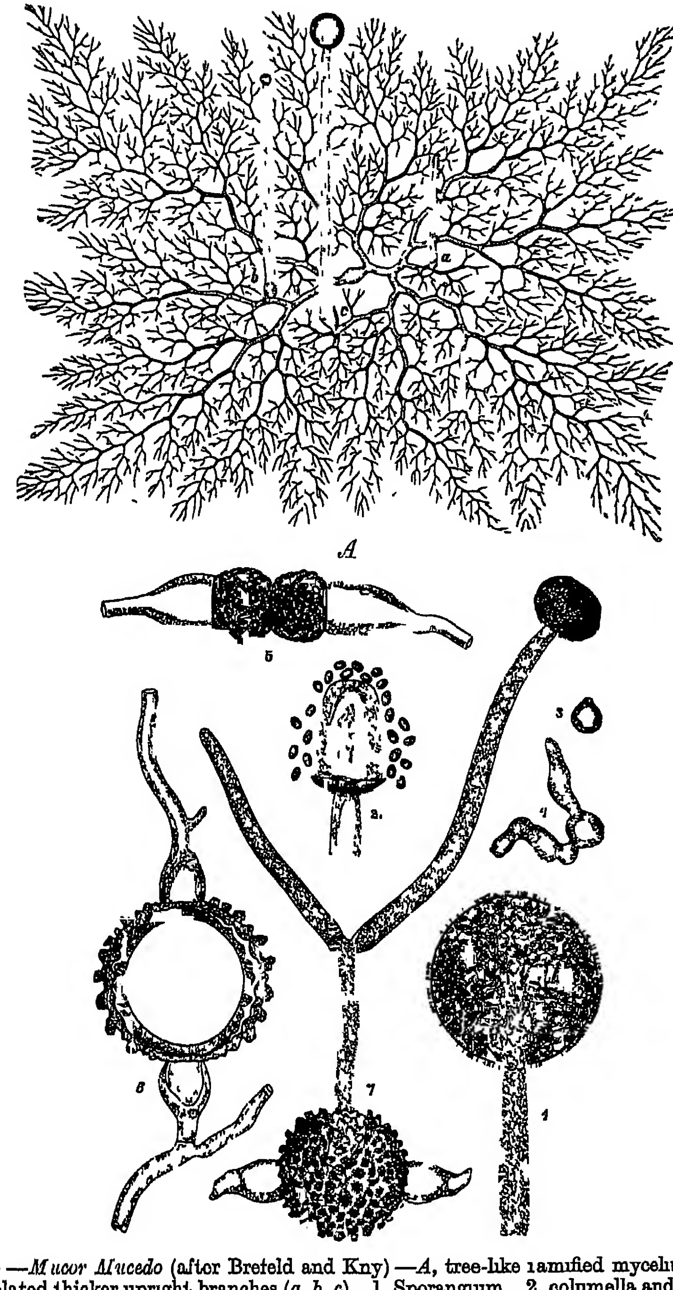


Fig. 35.—*Mucor Mucedo* (after Brefeld and Kny).—*A*, tree-like ramified mycelium with isolated thicker upright branches (*a*, *b*, *c*); 1, Sporangium; 2, columella and spores; 3, 4, germinating spores; 5, 6, development of the zygospore; 7, germinating zygospore with sporangium.

takes place by means of a conjugation Two short branches, filled with protoplasm and growing towards each other, form club-like swellings and come in contact at their free ends, which flatten out Each of the branches is then divided into two cells by a septum, and the end cells, which are in contact (the conjugating cells), coalesce by dissolution of the original double wall which separated them. The new cell thus formed—the zygospore —quickly increases in size and expands to a spherical shape (in *Mucor stolonifer* to a barrel shape), after which the wall thickens, and becomes stratified, externally it is dark in colour and covered with wart-like excrescences These outer layers are very resistant to the action of acids The contents possess an abundance of reserve stuff (fat) Cases occur where the zygosphore develops from a single cell without conjugation, and occasionally such a cell is formed at the tip of one of the mycelial hyphæ The zygospores are usually capable of germinating only after a long period of rest, the germ tube, after bursting the outer layers, quickly develops sporangia as described above Thus, in the zygospore we find a resting stage of the plant, an organ which by its structure enables the mould to preserve life during periods unfavourable to growth

These spores are usually formed only on the surface of the substratum with free access of air

The conditions on which the formation of zygospores depend have been exhaustively investigated by Blakeslee, who has shown that, in the case of the majority of species examined, it was essential that the conjugating hyphæ should belong to different individuals. In the case of *M Mucedo* it is necessary that these individuals should be derived from spores originating from different sporangia, otherwise no zygospores are formed In some species a difference could be detected in the structure of the two individuals which formed the zygospores In a minority of cases the zygospores were, however, formed from one and the same mycelium. Temperature affects their formation. thus in *M. Mucedo* they were produced at ordinary room temperature, but not at 26°-28° C. As a suitable medium, Saito and Naganishi used whey agar with the addition of 2 per cent. dextrose or steamed rice They could observe completely developed zygospores after the two mycelia, sown some distance apart, gradually drew nearer so as to form "zygospore lines," and at last united The crossing of two different though allied species also resulted in the formation of zygospores, which, however, could not be induced to germinate.

Mucor racemosus, which occurs on bread and decaying vegetable matter in very variable forms, has a branched, multicellular sporangiophore, which may also attain to a considerable height. Like *M. Mucedo*, the optimum temperature ranges from 20°-25° C. The brownish sporangia are developed at the ends of the branches.

The spores are colourless. Both the aerial and the submerged portions of the mycelium are capable of forming transverse septa, dividing the hyphæ into a number of short cells. These are usually filled with protoplasm, and assume a spherical or barrel shape, this was first observed by Bail They are termed **gemmæ.** The cells frequently form thickened cell-walls, and store up reserve food material, thus constituting a resting spore (**chlamydospore**). Both kinds of cells after separating from the mycelium may again vegetate under suitable conditions.

When free access is given to atmospheric oxygen, both spores and gemmæ germinate and from an initial germinal hypha develop into a mycelium. The case is different, however, when the development is going on in closed flasks, under these conditions, not only

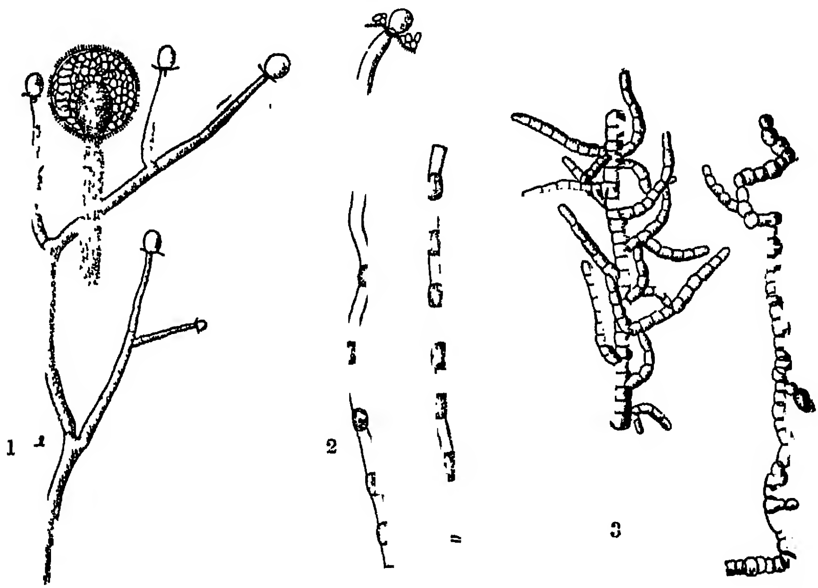


Fig 36 —*Mucor racemosus* —1, Branched sporangiophore and sporangia (highly magnified), 2, hyphæ with chlamydospores, 3, branched mycelium; gemmæ formation.

the spores and gemmæ, but even the normal mycelial hyphæ develop yeast-like budding cells, and thus form the "mucor yeast" or "spherical yeast." By cultivating *M racemosus* in a flask completely filled with wort, through which a stream of carbon dioxide is passed, a growth consisting exclusively of mucor yeast can be obtained In *M spinosus*, *M racemosus*, *Rhizopus nigricans* and *Thamnidium*, the yeast-like budding was found by Ritter to be prevented when oxygen was *entirely* excluded.

All species do not demand the presence of sugar as a condition governing the formation of yeast-like cells

Mucor erectus, with greyish-yellow transparent sporangia, which

may be found, for instance, on decaying potatoes, has the same microscopic appearance as *Mucor racemosus;* physiologically, however, it differs from this species.

Mucor circinelloides has a very characteristic appearance. The mycelium shows the remarkable branching which occurs in some of the species of *Mucor* The main branches send out short, root-like branches with frequent forking, at the base of these come new mycelial branches, which grow erect, and are able to form sporangia, the sporangiophore is sympodially branched. During its development it curls up, and to this fact the species owes its

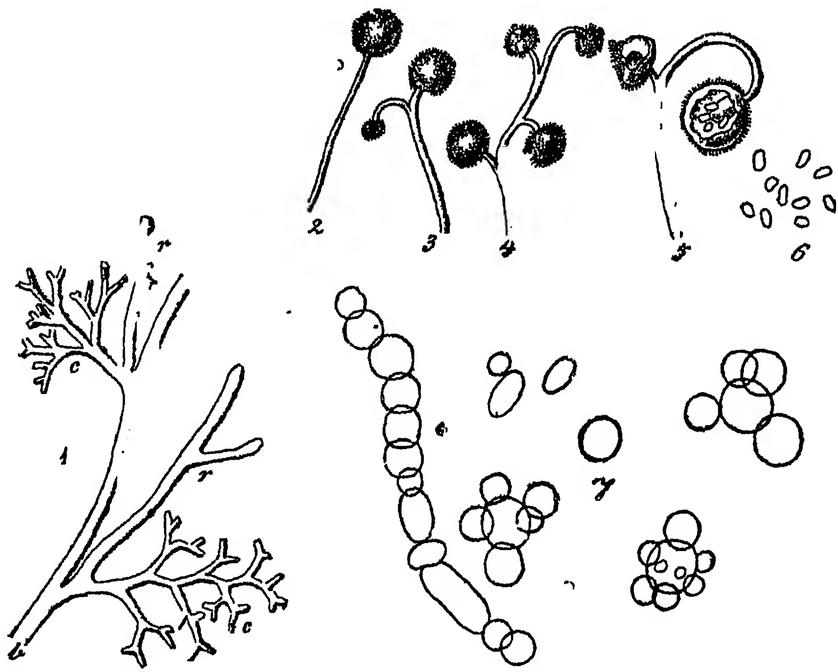


Fig 37—*Mucor circinelloides* (after van Tieghem and Gayon)—1, Mycelium, *b*, main branch; *c*, root-like branches, *r*, axillary branches, 2-4, development of sporangia, 5, opened sporangium, 6, spores, 7, submerged mycelium and budding cells.

name of *circinelloides.* In this form, like *Mucor spinosus*, the mycelium, when submerged in a saccharine liquid, produces gemmæ, similar in formation to those of *Mucor racemosus* and *Mucor erectus.* *Mucor spinosus* has a greyish-blue mycelium with spherical spores and brownish-black sporangium, which is distinguished by the uppermost part of the columella being studded with pointed, thorn-like protuberances.

Finally, *M. alternans* belongs to this group, and bears a close resemblance to *M circinelloides.* This fungus has the distinction of being the first of the *Mucor* species shown by Gayon

and Dubourg (in 1887) to possess the property of fermenting dextrin

The most interesting of all the species of *Mucor* is *M.* (*Amylomyces*) *Rouxii*, on account of its extended application to the hydrolysation of starch on a commercial scale. It was isolated by Calmette in 1892 from "Chinese yeast" small greyish-white cakes, consisting of rice grains kneaded together with various spices Calmette, however, only described the characteristic mycelium exhibiting gemmæ (chlamydospores), and called the fungus *Amylomyces* Wehmer subsequently described the sporangia, and Vuillemin described in detail its characteristic features On a solid substratum, such as rice, it forms a yellow covering, due to an aggregation of yellow oil in the cells The same appearance is observed when the mycelium spreads over the surface of liquids, but the submerged portions are grey in colour A temperature slightly above 30° C is best suited to its development. The mycelium frequently remains sterile The fructifying hyphæ vary greatly in length, the sporangia are small, almost spherical, and both they and the elliptical spores may be either light or dark in colour The fungus forms both gemmæ and "spherical yeast" Like most other species of *Mucor*, it has a tendency to vary morphologically

The diastatic enzyme reacts most powerfully at 35°-38° C., and produces chiefly dextrose. The process, carried on, as it now is, on a large scale in special factories, consists in first boiling the starch (maize or rice) under pressure, then liquefying the mass by the addition of small quantities of green malt or hydrochloric acid, and sterilising the liquid at a high temperature By adding a culture of spores at 38° C. the hydrolysation is rapidly effected.

Another species, *M Praini*, with similar characteristics, was isolated by Nechitch from Indian rice cakes. It has spherical sporangia, yellowish or dark brown in colour, and colourless spores of varying shape

A third species, isolated from Javan rice cakes, *M. javanicus*, has been described by Wehmer It forms a yellow growth on rice, produces a raised cushion of sporangia, yellowish-grey or light brown in colour. The sporangia are small, yellowish-brown, and transparent, whilst the spores are colourless, and of irregular shape. Like the former species, it is able to convert starch into sugar, and to bring about alcoholic fermentation.

Similar diastatic enzymes are met with in the species about to be described.

One of the most widely distributed members of the genus, differing considerably in form from the species already described, is *Rhizopus nigricans* (formerly known as *M. stolonifer*). The species attains considerable size, and very commonly occurs on succulent fruits. This mould is easily recognised, for its brownish-yellow

mycelium shoots out diagonally with thick hyphæ without septa. These attain a length of about 1 cm, then droop until their points touch the surface of the substratum, and shoot out fine ramified hyphæ resembling rootlets into the latter, whilst other hyphæ rise perpendicularly and develop sporangia, finally other branches form new runners The black spherical sporangium possesses a high, dome-shaped columella, contiguous to the broadened end of the sporangiophore, and develops a number of dark brown spores, round or angular. When these are freed by the absorption of the sporangium wall, the columella curves

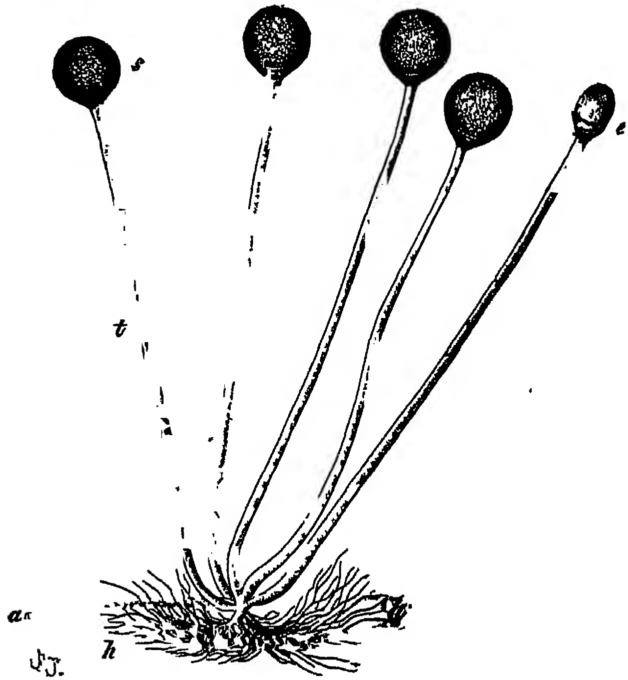


Fig 38.—*Rhizopus nigricans* (after Brefeld).—*a*, End of a runner or stolon; *t*, sporangiophore, *s*, sporangium, *e*, columella *h*, root-like hyphæ or rhizoids.

over the sporangiophore like an umbrella, the line of junction with the external wall remaining in evidence in the form of a collar. The fungus, when grown on very concentrated solutions of cane-sugar (49 per cent) at 18° C, was found by Bezssonoff to produce abundance of gemmæ and zygospores Zygospores are produced by the fusion of hyphæ, which, according to Blakeslee, belong to different mycelia *Rh nigricans* occurs on a great variety of juicy fruits, causing them to decay, and thus working considerable havoc. Behrens has shown that the damage is caused through

the secretion by the fungus of a poisonous substance, which kills the fruit cells. It is also of frequent occurrence on malt.

A similar species, *Rh. Oryzæ* (*Chlamydomucor Oryzæ*) was discovered on Javan rice cakes by Went and Prinsen Geerligs (1895). On account of its power of hydrolysing starch, it is employed in the preparation of arrack from rice. It produces large numbers of gemmæ. A specially interesting form is *Rh. japonicus*, which, like *M. Rouxii*, is applied industrially to the hydrolysation of starch, more especially of maize starch. It was isolated by Boidin in 1900 from Japanese koji, and, like the species discovered by Calmette, was called *Amylomyces* (β); it was described more exactly by Vuillemin. It shows a great resemblance to both the former species, and, like *Rh. Oryzæ*, forms gemmæ. Vuillemin also described *Rh. tonkinensis* (*Amylomyces* γ), which has the same structure as the foregoing, but reacts differently on the sugars.

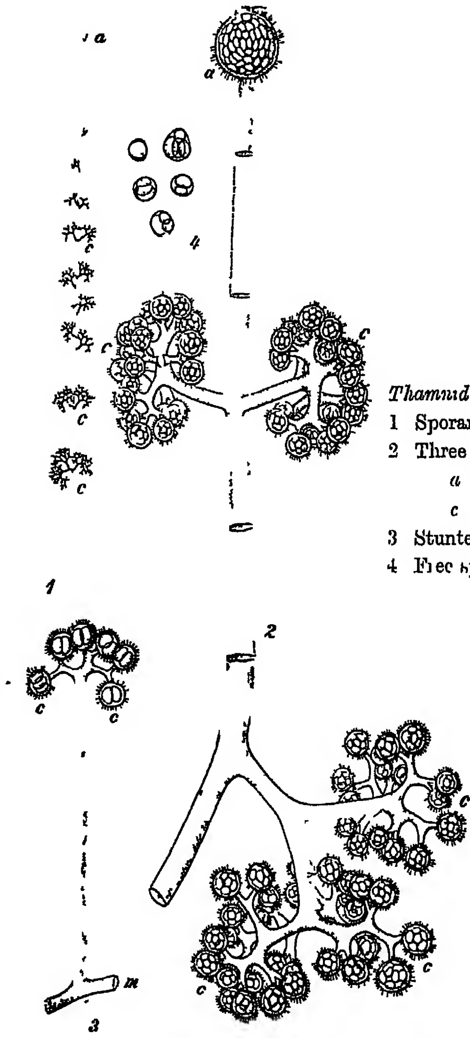
Rhizopus Delemar, introduced later by Boidin, has been employed in the Amylo process. Its growth and diastatic power may be illustrated by the fact, established by Boidin, that the spores from a one-litre Pasteur flask are sufficient to saccharify the starch in a vessel of 1,200 hectolitres capacity. Morphologically, it bears a close resemblance to *R. nigricans*. It was described in detail by Hanzawa and Usomi. Physiologically, it differs from the latter species, not only in the property described, but also by not growing at temperatures below 12° C., neither will it stand such high temperatures as its fellow-organism (about 42° C.). Optimum 25° to 30° C. It ferments saccharose, glucose, mannose, inulin, galactose, fructose, maltose, and raffinose, and in unhopped wort was found to yield 2.7 per cent. of alcohol by weight. It produces free acids.

Amongst other moulds standing in close relationship to the *Mucor* species, *Phycomyces nitens* is frequently referred to in the literature. It usually occurs on oily substrata, but also on bread, excrement, etc. It resembles *Mucor*, and its olive green sporangiophores with their metallic lustre attain to an extraordinary size. The sporangium is black, the columella pear-shaped, and the spores yellowish.

Thamnidium elegans, frequently occurring on the dung of various animals and on breadstuffs, is a fine mould with sporangia recalling those of *Mucor*; but in addition to the terminal sporangium formed at the apex of the main sporangiophore, the latter gives rise to a number of forked side branches, on which sessile sporangia (sporangioles) are formed without columella and with fewer spores.

In conclusion, we may allude to *Sporodinia grandis*, a fungus often met with on toadstools growing in woods. It forms a dense felt of branching hyphæ with numerous sporangia, and also produces zygospores.

Hansen's determination of the limits of temperature for the three species, *M racemosus*, *neglectus*, and *alpinus*, when grown on wort-agar gelatine and in wort, proved that the formation of sporangia and zygospores can proceed at a slightly lower maximum



Thamnidium elegans (after Brefeld)

1 Sporangiophore (low magnification).

2 Three sections of above (high magnification)

a Terminal sporangium

c Sporangioles

3 Stunted fruit carriers with sporangioles.

4 Free sporangioles

Fig 39

than is required for vegetative growth (the behaviour is thus analogous to that of the Saccharomycetes) The development of sporangia can, however, proceed at the same minimum as that required for vegetative growth. The species vary with regard to the

temperature limits for sporangia, on the one hand, and for zygospores, on the other; thus *M. alpinus* exhibits a higher maximum for the formation of sporangia than of zygospores, but the reverse is the case with *M. neglectus*. It follows that the temperature limits may serve to determine the species. *Mucor racemosus*, for example, when grown on the media alluded to, is limited for vegetative growth to a maximum of 32°-33° C., and a minimum of 0·5° C.; *M. alpinus* to a maximum of 29°-31° C., and a minimum of 0·5 C.; *M. neglectus* to a maximum of 33° C., and a minimum of 3° C.

The *Mucor* species are of special interest to us, because they can act, in varying degree, as true alcoholic ferments. Their fermentative power is not connected with the formation of budding gemmæ, for these have not been observed in either *Mucor Mucedo* or *M. stolonifer*. This form of fermentation has been regarded as a special kind of breathing. Unlike normal respiration, such as is performed by every organised being—the inhalation of oxygen and exhalation of carbon dioxide—it can take place in the absence of free oxygen. The oxygen in the cell contents makes fresh intra-molecular linkings, with the result that the carbohydrates, and more particularly the sugars, become disintegrated, so that not only carbonic acid but also alcohol is produced. Adopting the term suggested by Pfluger, the process is known as intramolecular respiration. This conception implies that the fermentative change produced by *Mucor*, which is only possible in the absence of free oxygen, differs essentially from that brought about by yeast, which can proceed either in the presence or absence of free oxygen. Wehmer's experiments with two species of *Mucor* (*M. racemosus* and *M. japonicus*) have shown, however, that the production of alcohol was not diminished by the constant bubbling of air through the liquid, nor yet when the fermentation is carried out in very thin films of liquid with a large surface exposed. In other directions the two kinds of fermentation possess characteristics in common, and the collective evidence makes it difficult to regard the processes as essentially different. On the other hand, Palladin and Kostytschew, and others, have proved that the two fermentations are not identical. A special alcohol enzyme, such as that isolated from yeast, has not been isolated from *Mucor* mycelium. Kostytschew observed, however, that the mycelium of *M. racemosus*, which had been killed by treatment with acetone, was able to produce an amount of carbon dioxide equal to that evolved by the living cells. *M. racemosus*, Hansen's *M. neglectus* and (according to Saito) *Rhizopus japonicus*, var. *angulosporus*, and *Rh. Tamari* are the only species capable of inverting and fermenting a cane-sugar solution. This was proved by Fitz for *M. racemosus*, and confirmed by Hansen and others. Kostytschew made the interesting observation that among the individual races of *M. racemosus* it is possible to point out well-marked physiological differences, whilst

morphologically they are identical. One race (+), which under parallel conditions shows the more vigorous development, cannot invert saccharose, whereas this sugar is inverted by the other, more feebly developed race (—).

The great majority of species are able to ferment maltose, invert sugar, and dextrose.

Considerable diversity may be observed amongst the species in regard to the production of alcohol. The same rules which govern yeast fermentation seem on the whole to apply to these processes. Thus, according to Wehmer, when the general conditions are favourable, in presence of oxygen and at a medium temperature, the fermentation is practically completed in the course of a few days. A remarkable feature of the fermentation produced by these fungi is that the liquor remains clear throughout the operation.

Some of the results obtained during Hansen's investigations may be quoted to show the difference in the productivity of the various species

M erectus possesses the greatest fermentative activity. In beer-wort (14°-15° *Balling*), it yields up to 8 per cent. by volume of alcohol It also induces alcoholic fermentation in dextrin solutions, and hydrolyses starch. *Mucor spinosus* yields up to 5·5 per cent by volume of alcohol in beer-wort. In maltose solutions distinct fermentation phenomena were observed, and after the lapse of eight months the liquid contained 3·4 per cent. of alcohol *Mucor Mucedo* has a comparatively feeble fermentative power both in wort (up to 3 per cent. of alcohol) and in maltose and dextrose solutions *Mucor racemosus* produces as much as 7 per cent of alcohol in wort, secretes invertase, and ferments the invert sugar

According to Gayon, *Mucor circinelloides* reacts powerfully on invert sugar (yielding 5·5 per cent. by volume of alcohol). According to Wehmer, *M. javanicus* produces 4 to 5 per cent. of alcohol in a few days.

Concerning the formation of **acid** from sugar solutions, we may note Wehmer's observation of the production of citric acid by *M. pyriformis* Goupil found that *Am. Rouxii* was able to convert a considerable proportion of the sugar in solution (up to 25 per cent.) into succinic acid, the amount depending on the acidity of the solution *Rhizopus chinensis* (Saito) produces lactic acid. Oxalic acid is produced by several species Most species liquefy gelatine, but quite slowly as a rule Albumen-splitting enzymes occur in the various species, and some appear to play a part in the ripening of cheese

5. Monilia.

A number of different fungi of comparatively simple structure are described under this name in works on mycology. From a mycelium, the colour of which varies according to the species, branches are thrown up which give rise to series of oval or elliptical spores. The genus has an interest for us on account of one of its species, named by Hansen *Monilia candida* from Bonorden's description, which possesses very remarkable physiological properties. It occurs in nature in the form of a white layer covering fresh cow-dung, and on sweet, succulent fruits. When introduced into wort, it develops a copious growth of yeast-like cells. At the same time it excites a vigorous alcoholic fermentation, and whilst this

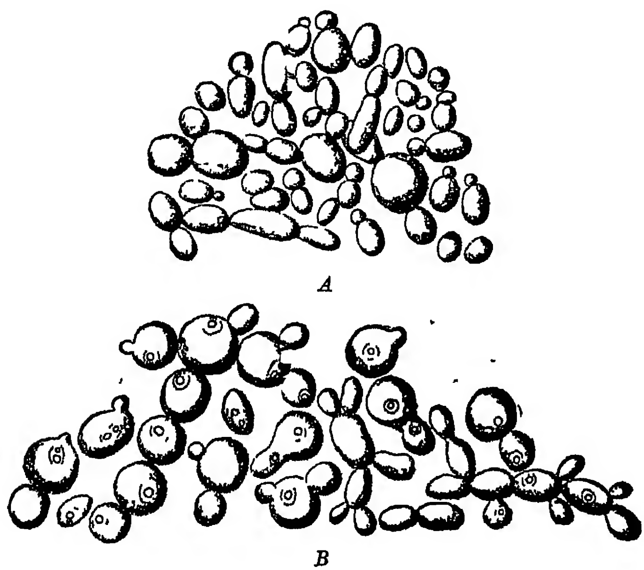


Fig. 40.—*Monilia candida* (after Hansen)—*A*, growth in beer-wort or other saccharine nutritive liquids, *B*, cells of a young film-formation

is progressing forms a mycoderma-like film on the liquid, the cells in this film extend further and further, and finally form a complete mycelium. During the early fermentation the fungus produced only 1·1 per cent by volume of alcohol, whilst *S. cerevisiæ* gave 6 per cent.; but the *Monilia* continued the fermentation, and produced at the end of six months 5 per cent. by volume of alcohol, whilst the culture yeast gave no further quantity.

Hansen states that *Monilia* does not secrete invertase, but, nevertheless, ferments cane-sugar, from which he concludes that cane-sugar is directly fermentable He suggested, however, the possibility that cane-sugar may be converted into invert sugar in

the interior of the cells, and that the latter is immediately fermented

Hansen's observations were confirmed by the work of E Fischer

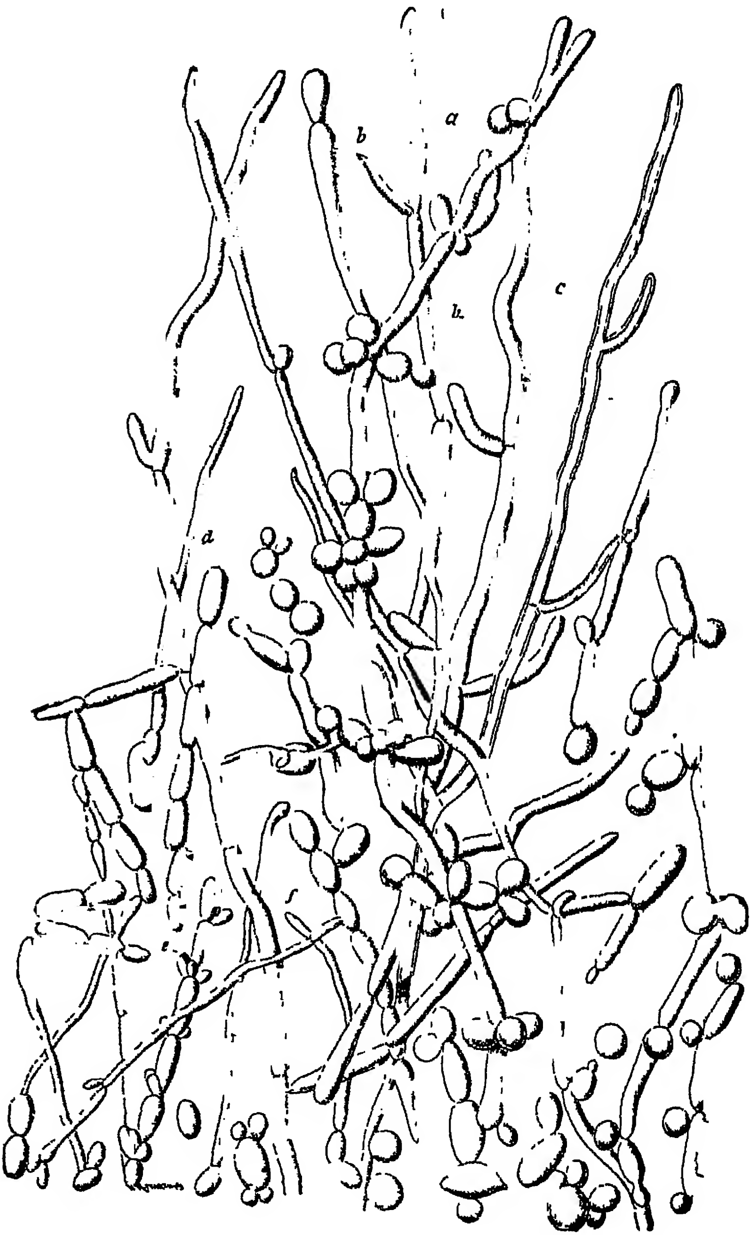


Fig 41

and P. Lindner, and subsequently by Buchner and Meisenheimer. They proved that an inverting enzyme cannot be extracted either from the fresh or from the dried vegetation. On the other hand, they were able to obtain preparations which inverted cane-sugar actively, either by grinding the cells with powdered glass, by killing them with acetone or by pressing out the juice (see Chap. v.). Thus the fungus contains an inverting enzyme, but it is completely retained by the protoplasm of the living cell. In contrast with yeast invertase, *Monilia* invertase is insoluble in water; it does not diffuse, as yeast invertase does, through the cell-wall, or through the protoplasmic lining of the cells, neither does it diffuse through parchment.

According to Fischer, maltose is split up both by fresh and by dried *Monilia*, and also by an aqueous extract of a dried growth; he therefore infers that *Monilia* contains the enzyme maltase discovered by him in *S. cerevisiæ*.

Fig 41.—*Monilia candida* (after Hansen).—Mould growths like *a* are frequent, they consist of chains of elongated cells, more or less thread-like, and rather loosely united; at each joint there is generally a verticil of oval cells, which readily fall off, *b* represents another form, also of frequent occurrence, but distinguished from the former by having no verticillate cells; instead of these there generally issues from every joint a branch of the same form as the mother cell, but shorter; the links of these chains are often closely united, the constrictions in many cases disappear, and a very typical mycelium, with distinct transverse septa (*c*) is produced, the forms *b* and *c* occur in the nutritive medium, *a* commonly on the surface. Forms like *d* have much resemblance to *Oidium lactis*. *e* shows a train of pear shaped cells with verticils of yeast-cells resembling *S. exiguus*; the chain of lemon-shaped cells represented at *f* closely resembles Ehrenberg's figures of *Oidium fructigenum*. Between the principal forms described there are numerous yeast-cells of different forms, variously arranged in colonies.

According to Bau, *Monilia* also ferments dextrin formed by diastase.

A certain amount of carbon dioxide and ethyl alcohol is developed in liquids undergoing a *Monilia* fermentation.

Finally, this fungus is distinguished by its power of withstanding high temperatures. In beer-wort and cane-sugar solutions it develops vigorously at 40° C., and induces an active fermentation at this temperature. The limits of temperature for the development of *Monilia* in wort are, according to Hansen, maximum 42°-43° C., minimum 4°-6° C.

A very interesting species, *M. vini*, was described by Osterwalder, who found it growing freely in fermented apple wine. It forms on gelatine colonies, which at first grow like common wine yeast and afterwards develop fringed branches. In fermenting liquids it forms a compact layer on the bottom, on which mould-like flakes develop later on; they contain ellipsoidal and long cells with peculiar angular vacuoles. The mould filaments are

ramified, mostly with no septa, between them are found larger tree-shaped, loose cell groupings It is a vigorous ferment—more vigorous, indeed, than any other species of this genus. It completely ferments fruit- and grape-wine, even of high acidity, it is also capable of developing in fermented wines and fermenting the residual sugar In the course of fermentation it forms volatile and non-volatile acids (including lactic acid) The sugars most readily fermented are dextrose and lævulose, next come saccharose, lactose, galactose, and, finally, maltose, which is but weakly fermented It secrets invertase by contrast with *M. candida*

Many other species have been described, amongst which may be mentioned *M sitophila*, discovered by Went, which grows on the earth nut (*Arachis hypogaea*) in West Java Its mycelium extends by degrees throughout the entire fruit, the hyphæ assuming a yellow colour on exposure to air By means of the various enzymes which the fungus contains, a change is brought about in the fruit contents In this fermented condition the earth nuts are eaten in large quantity by the natives *Sachsia suaveolens*, discovered by P Lindner, is also an interesting fungus belonging to this group It produces a high percentage of alcohol in wort, and develops a wine bouquet *M variabilis*, described by Lindner, is distinguished by an extraordinary multiplicity of forms.

6. Oidium (Oospora) lactis.

Oidium lactis is a mould which has played an important part in the literature of the physiology of fermentation, and in that of medicine. It is known as the milk mould

The transparent, thin-walled hyphæ, often forked and branched, form a thick white felt, in the upper part of the filaments transverse septa are formed close together, after which the single cells, filled with very refractive protoplasm, are detached as conidia, in longitudinal section they are rectangular with rounded corners. In growth of this mould, spherical, oval, pear-shaped conidia, and others of quite irregular form are, however, almost always present. These organs of propagation, the only ones known, send out one or more germ tubes. When the fungus grows on solid substrata, the hyphæ unite and form remarkable conical bodies.

Fresenius correctly gave to this species the specific name of *lactis*, for universal experience shows that it has its ordinary habitat in milk, where it may usually be found. It also occurs spontaneously in various other liquids, and among these in the saccharine liquors which are employed in the fermentation industries in the latter it is able to induce a feeble alcoholic fermentation. Thus, according to Lang and Freudenreich, it produces in milk and grape-sugar solutions, in the course of about ten days, 0·55 per cent., and in five weeks, 1 per cent by volume of alcohol;

smaller proportions of alcohol are produced in cane-sugar and maltose solutions. Its maximum temperature is, according to Hansen, 37·5° C., and its minimum below 0·5° C Cultures made

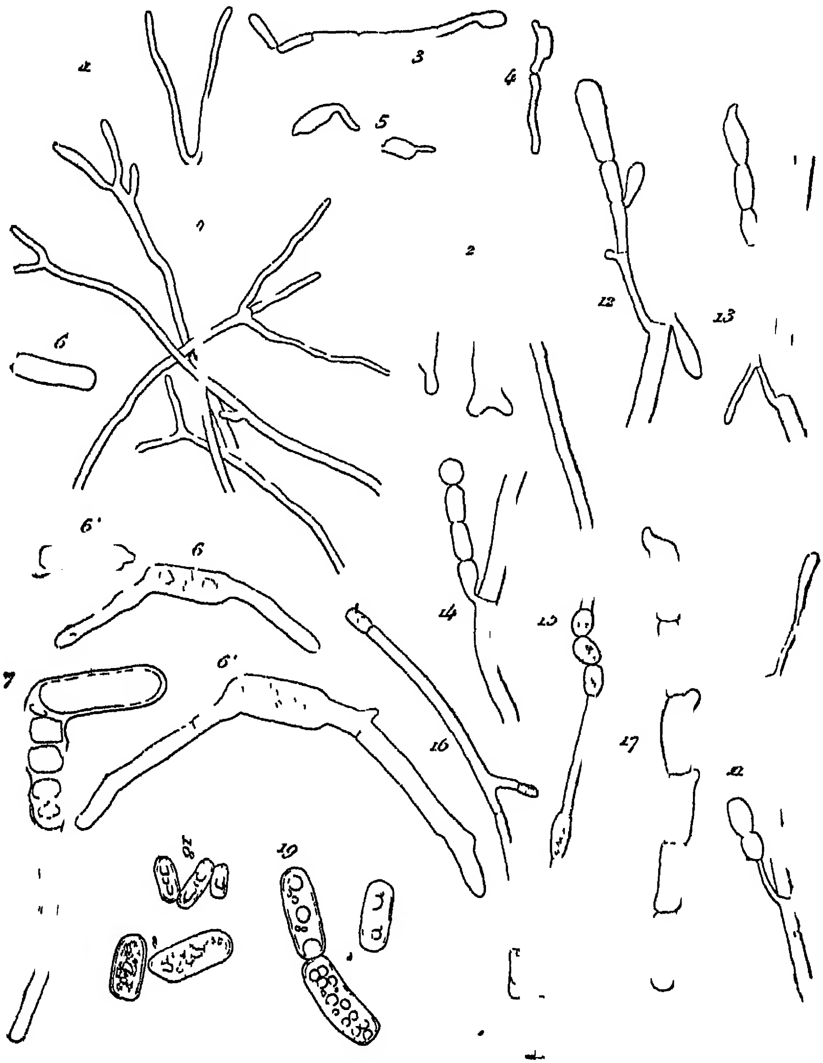


Fig. 42—*Oidium lactis* (after Hansen)—1, Hyphæ with forked partitions, 2, two ends of hyphæ—one with forked partition, the other with the beginning of a formation of a spherical link, 3-7, germinating conidia, 6-6‴, germination of a conidium sown in hopped beer-wort in Ranvier's chamber and represented at several stages; at each end germ tubes have developed after nine hours (6‴) these have formed transverse septa and the first indications of branchings; 11-14, abnormal forms, 15, 16, hyphæ with interstitial cells, filled with plasma, 17, chain of germinating conidia, 18, conidia which have lain for some time in a sugar-solution, the contents show globules of oil; 19, old conidia

in lactose nutritive solution develop a powerful odour, resembling that of soft cheese, such as Limburg cheese. It is believed that *Oidium* is of importance in the ripening of several kinds of cheese, because it absorbs the acids produced by lactic acid bacteria, and thus paves the way for peptonising bacteria Casein, in sterilised milk, is rapidly decomposed by the fungus. According to O. Jensen, an *Oidium* is always present in rancid butter.

The fungus may occur in beer, especially when poor in alcohol. As the amount of alcohol increases, the conditions for its growth become less favourable, still, neither wort nor beer is exposed to the danger of being attacked to any extent by *Oidium*, since it is not able to compete in the struggle for existence with the crowd of organisms which at once appear when such fermentable liquids are exposed to the atmospheric germs

In numerous investigations with top-fermentation yeast, the author has found that it offers a very favourable nutritive material for this fungus, especially when the yeast is in a quiescent state at the end of the fermentation. Sometimes a microscopic examination has shown an enormous number of conidia It is not known what influence such a growth exercises on the quality of the yeast and the beer, but without doubt it is advisable to avoid the fungus as much as possible It forms vigorous growths on pressed yeast also, which have a deleterious action on the quality of the yeast.

A large number of species and varieties find shelter under the name *O. lactis*. Weigmann has described several, and Grimm also isolated a number of forms from sour milk, cheese, etc., which differ clearly from each other both in regard to their characteristic growths on gelatine, and especially on potatoes, and also in regard to their peptonising action on the substratum

Schnell also examined numerous growths from widely varying media, and distinguished a number of races showing marked variation, particularly at temperatures near the optimum—25° to 30° C. They vary in size, appearance on liquid and solid media, power of liquefying gelatine and splitting up fat, and fermentative activity. All the races were able to produce and consume acid, to break down albuminoids to free ammonia, and to assimilate ethyl alcohol The races occurring on living fruit are capable of decomposing the albuminoids and the cell-walls. Several of the species examined tended to destroy pressed culture yeast in a quiescent state, both the membrane and the albuminoid constituents of the cell were attacked, the latter yielding free ammonia. Fermentation was apparently arrested by these forms All growths isolated from milk could decompose it, producing a marked cheesy smell. Thus, they belong to the cheese ripeners properly so-called. Among them Schnell distinguished one race which he says should be described as a species, *O caseı*, the development of which is

peculiar in that, without forming a mycelium, it produces a whole series of regular oval oidia, which are linked in chains. On 2 per cent. wort-agar it forms a yellow, smooth, moist covering. On sweet wort it develops at the optimum (23° to 25° C) a mouldy, yellowish, translucent growth, which falls to pieces if shaken

An aroma-forming species, *O. suaveolens*, was found by Krzemecki in water. It evolves a powerful odour of fruit-ethers and grows in beer-wort, yeast decoction, fruit juice, etc Morphologically it closely resembles *O. lactis*, from which it may be distinguished by its growth on beer-wort, where it forms a white, fluted film and a thick layer on the wall of the vessel Optimum for growth is at 25° to 27° C. The aroma is formed specially in nutrient liquids containing maltose and dextrose. It yields traces cf alcohol and acid

7 Endomyces.

The reason why this genus is described here is that several of its species bear some resemblance to Oidium in their morphological characteristics. By reason of their endogenous spore-formation they appear to be allied to the Saccharomycetes. The mycelium is ramified, with septa, and is sometimes divided like that of Oidium. it forms conidia by budding from the hyphæ, and in some species also chlamydospores The cells which are set free develop by germinating filaments, budding or division In most species, asci were observed, which are formed as lateral branches or buds on the hyphæ, or from some articles of these, or by the fusion of two cells. The asci contain up to four spores each.

The species first discovered, *E. decipiens*, was described by Tulasne, later by de Bary and Brefeld It shows a well-marked formation of oidia and chlamydospores, on short lateral twigs it forms asci with four hat-shaped spores resembling those of *Willia anomala*. The cells develop germinating filaments, but no buds This species has no fermenting power

E Magnusii, from the gum of oak trees, was described by Ludwig, later by Brefeld, Guilliermond and Rose Its asci contain four oval spores with a double membrane, its episporium has wart-like protuberances Spore formation can be observed by culture on potato slices The cells develop germinating threads, but no buds Brefeld observed no fermentation; according to Rose, it ferments dextrose, fructose, saccharose, mannose, and raffinose It assimilates maltose

E fibuliger was found and described by Lindner and also by Dombrowski It was so named on account of the peculiar fusion of the mycelium filaments. It forms conidia resembling raisin-pips, elongated yeast cells and globular asci containing hat-shaped spores with two membranes and a smooth surface The cells develop

both germinating filaments and buds. Lindner found that it fermented dextrose and fructose, inverted and fermented saccharose, but did not react with maltose.

E. Javanicus was described by Klocker It will grow in dilute wort, and forms a mycelium with oidium-like chains and yeast cells, mainly lemon-shaped, others being globular, ellipsoidal, and elongated The cells form both germinating filaments and buds Spores develop in the mycelium as well as in the yeast cells, they are ellipsoidal or take the form of a flattened sphere; they have one membrane, provided with small protuberances, and round the middle of the spore there is a very prominent collar. This species does not ferment dextrose nor does it invert saccharose.

E. albicans (*Oidium albicans*) forms yeast cells and chlamydospores; in each ascus Vuillemin observed four flattened, kidney-shaped spores. It coagulates milk and weakly ferments dextrose. It is a pathogenic organism.

E. Lindneri was detected by Saito in the so-called Chinese yeast, which is used for making Chinese millet beer From the mycelium spring conidia, which shoot germinating filaments, the mycelium forms asci with two to four hat-shaped spores, having two membranes and forming germinating filaments. It ferments glucose, fructose, mannose, maltose, saccharose, and raffinose (feebly), also dextrin

E. Hordei, likewise described by Saito, has hat-shaped spores with two membranes. It gives an active fermentation in wort and koji decoction, and physiologically bears some analogy to *E. Lindneri*.

E vernalis (Lindner) occurs in the milky discharge of trees budding in spring. It will grow freely on thin layers of a saccharine medium, which, however, it does not ferment. Cells grown with access of air form abundance of fat

E. mali (Lewis), which bears a resemblance to *E fibuliger*, is found on acid media and causes apples to rot.

8. Fusarium.

The red colour occasionally occurring on malt grain is due to various fungi, among which is a *Fusarium* described by Matthews and Klein. The mould formation begins on the germinating part of the grain, and spreads thence over its surface The filaments of the mycelium, which show globular swellings, are connected by numerous bridgings The red colouring matter is present in the contents of the filaments. On a moist medium the membranes gradually swell, forming a slimy envelope, which is coloured violet by iodine. The oval conidia either germinate directly, or first grow into sickle-shaped septate cells. Germinating filaments issue from the points of the latter, and by slow degrees the cells swell

out. Both the mycelium and the sickle-shaped conidia are able to produce thick-walled spores like gemmæ The fungus does not appear capable of hindering the growth of sound malt grain, even if the mycelium spreads freely over its surface Generally speaking, it only attacks diseased grain.

9. Chalara.

Chalara Mycoderma is described in Pasteur's *Études sur la bière* as one of the organisms commonly occurring on grapes The mycelium forms a film on liquids, and consists of branched, greyish

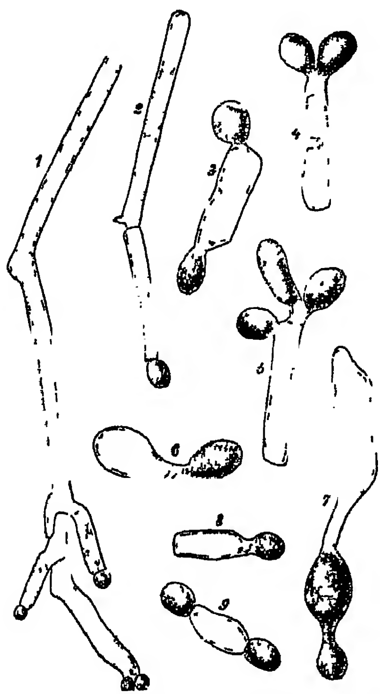


FIG 43—*Chalara Mycoderma* (after Hansen)—1, A branched hypha, the terminal limb of which is throwing off conidia, 2, a hypha, at the upper cell of which a sterigma, which has thrown off conidia, 3-9, various forms of hyphæ links, which are separating conidia

filaments, which at different points develop conidia of unequal form and size filled with glistening and highly refractive protoplasm. The mycelium frequently divides up into separate *Oidium*-like cells. Cienkowski first gave a detailed description of *Chalara* in his memoir on the film-forming fungi. Hansen found that this mould develops both in ordinary wort and lager beer, as well as in the diluted liquors.

10 Dematium pullulans.

A mould about which a great deal has been written in the literature of our subject is *Dematium pullulans*, which was first described by de Bary, and more minutely by Loew. It frequently occurs on fruits, especially grapes, and has a branched mycelium from which buds are thrown out, these have a striking resemblance to ordinary yeast cells, and are able either to propagate through

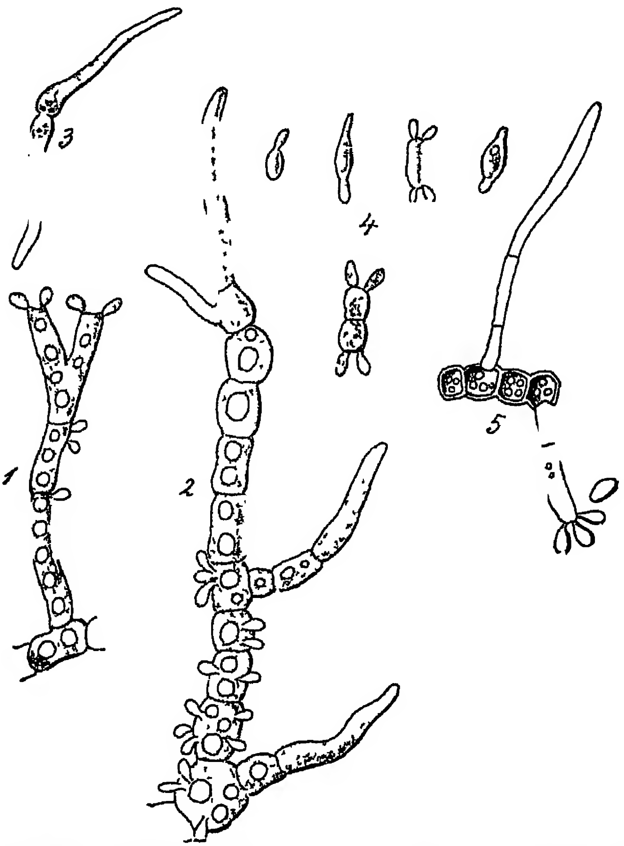


Fig 44—*Dematium pullulans* (after Loew)—1, 2, Full-grown mycelial threads with yeast-like cells, 3, cells of the latter developing to mycelial threads, 4, cells with yeast-like buds; 5, appearance of yeast-like cells on the germ tubes of the cells with brown covering

many generations, by yeast-like budding, or to produce germinating filaments giving rise to a mycelium. Skerst states that the mycelium develops more particularly at low temperatures, whereas the yeast-like cells form at temperatures of 19°-32° C In a strongly concentrated grape-sugar solution the fungus chiefly develops mycelium When this has attained a certain age, it forms numerous,

closely contiguous, transverse septa, and gradually turns brown or olive green, this forms the resting stage of the plant In Hansen's air analyses, *Dematium* was frequently found from spring until late autumn in wort to which air had access. He observed that when the mould was sown in a saccharine liquid it at first developed only mycelial filaments, after some time, however, yeast-like cells separated, without inducing alcoholic fermentation

P Lindner states that one *Dematium* species produces a ropiness when cultivated in wort, owing to the formation of slime from the cell membrane. *Dematium* species are also found in milk and dairy

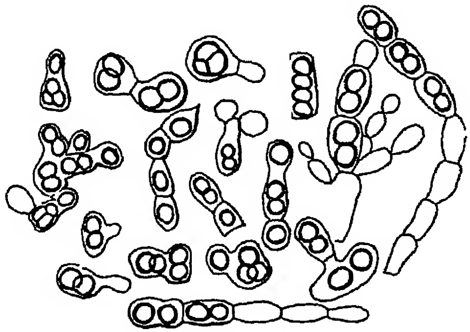


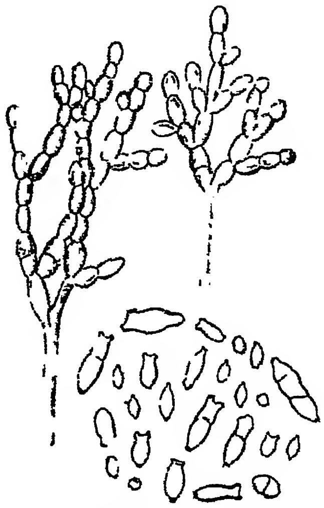
Fig. 45 *Dematium species* (Jorgensen) —Spore-formation in mycelial threads

products. A great development of *Dematium* occurs in the sap which oozes from the cut stem of the vine and, according to Wortmann, this is the main reason why the sap is gradually converted into a slimy, glutinous mass Wine must may also turn slimy, for the same reason, if it is allowed to ferment too slowly In isolated cases Wortmann observed that *Dematium* exercises a destructive influence on grapes In 1895 the author observed endogenous spore-formation in *Dematium*-like moulds occurring on dried grapes, but the organism showed no development of the resting cells described above The spores developed nothing but a yeast growth in saccharine liquids The yeast thus developed was capable of spore-formation, and is, therefore, a true *Saccharomyces*

11. **Cladosporium herbarum** (Fig 46)

This mould occurs along with others in fermentable liquids, in the fermenting rooms, and also on hops, malt, etc It sometimes occurs in very large quantities in the fermenting rooms The author found, in one case, that the ceiling and a portion of the walls of a bottom-fermentation room were thickly covered with small black patches; these consisted of *Cladosporium*, the conidia of which were consequently always present in the yeast The plant

consists of a yellowish-brown mycelium with short, straight filaments, stiff and brittle, those growing erect can produce at their upper extremities conidia of very varying form—spherical, oval, or cylindrical, straight or curved In contrast to *Penicillium*, where the new conidia are formed basi-petally (*i e.*, below those already cut off), in the case of *Cladosporium*, they spring either apically or laterally from a budding of the mother conidium, the development thus being basifugal. The name *Cladosporium herbarum* doubtless includes several closely related species According to Janszewski's researches, the same species may occur in different forms and with a varying size of cell He showed that the commonly occurring species represents a stage in the development of an Ascomycete (*Mycosphaerella*), the perithecia of which bear some resemblance to those of *Aspergillus*.



ıg 46 — *Cladosporium herbarum* — Conidia-forming hyphæ (Loew) and Conidia (Holm)

Wortmann includes *Cladosporium* amongst those fungi, the mycelium of which, growing through the corks of wine bottles, give rise to the corked flavour of wine These and other species of fungi occur during the ripening of cheese which, through their development, acquires a dark brown or black colour. Fungi belonging to this group play a part in bringing about the decomposition of eggs Zopf has identified a species which recent investigations have shown to be capable of forcing germinal hyphæ through the egg shell and membrane, and of gradually decomposing the albumen. O. Jensen has observed that a species f *Cladosporium* promotes the rancidity of butter on account of s power of splitting up fats Eriksson states that rye is sometimes ttacked by *Cladosporium*, and that the mould when consumed in re bread or in beer may prove pathogenic

Concerning these, or certain closely related forms, Zopf detailed xact morphological research, accompanied by numerous illusations, in his memoir on *Fumago*, and also in his work on the ngi. This black, soot-like fungus occurs very frequently on ants. Frank correctly says —" We are still quite in the dark ith regard to specific differences, due especially to the frequent lymorphism of these organisms, and to the fact that the different olution forms are scarcely ever found together "

The "**Black Yeasts,**" mentioned in the literature, *Saccharomyces ger*, *Torula nigra*, outwardly bear some resemblance to *Clado-*

sporium. According to researches made by Noldin (communicated by Will), they develop a slightly ramified mycelium originally colourless, but afterwards turning olive-green, dark-green, or black, with ellipsoidal, oval, spherical, or apiculatus-like conidia, single or piled on a broad base They form groupings which in some species or varieties consist of single lines, in others ramified. They sometimes grow out to form hyphæ, or multiply by budding Some conidia develop as gemmæ with thickened walls No endogenous spore-formation has been found They are not very resistant to acids, and do not yield alcohol

Among the various fungi occurring on the vine, the two following parasites have obtained an unenviable notoriety, on account of the great damage they cause —

12 Oidium (Erysiphe) Tuckeri.

This fungus, which is also called "the true mildew," forms whitish spots, changing to brown, on the leaves and shoots of the vine These consist of mycelial filaments, from which elliptical or oblong, colourless conidia separate, 8 μ long and 5 μ thick. The mycelium spreads over the fruit, which is gradually covered with a delicate growth of a grey colour, while it thrusts through the fruit skin roundish suckers, killing the epidermal cells. When grapes are attacked at an earlier stage, the epidermis is unable to keep up with the growth of the contents, it then gradually splits open like skin affected by scurf, the contents exude, and the grapes either dry up or putrefy They may impart to wine a very unpleasant smell and taste

On the full-grown grapes the fungus does not do so much harm, but may still prevent the further maturing of the fruit The best remedy for this dangerous parasite is sprinkling with finely powdered sulphur, but this only takes effect in sunny weather.

13 Plasmopara (Peronospora viticola).

The second vine fungus is "the false mildew," *Peronospora viticola*, which penetrates to the interior of the leaves and fruit, where it spreads and kills the cells The conidiophores burst out from the stomata of the leaves in tufts The upper part is branched, and both the branches and the principal axis end in short conical apices The conidia are oval, 12 to 30 μ long, and have a smooth, colourless membrane In the conidia, as a rule, five or six swarming spores are formed, which burst out when the conidia are immersed in water. According to Muller-Thurgau (*Landw. Jahrb d Schweiz*, 1912) the germ tubes of the swarming spores are unable to pierce the epidermis of the leaves, and consequently penetrate through the stomata. Thus, the infection of the leaves takes place only

from the under side. The growth forms thick, prominent whitish spots on both leaves and fruit. In the interior of the plant, big, globular oospores are formed (30 μ diameter), which have a brownish membrane, smooth or slightly fluted, and are surrounded by the thin, colourless, or yellowish oogonium wall. This fungus causes great injury, because the grapes either wither away or putrefy

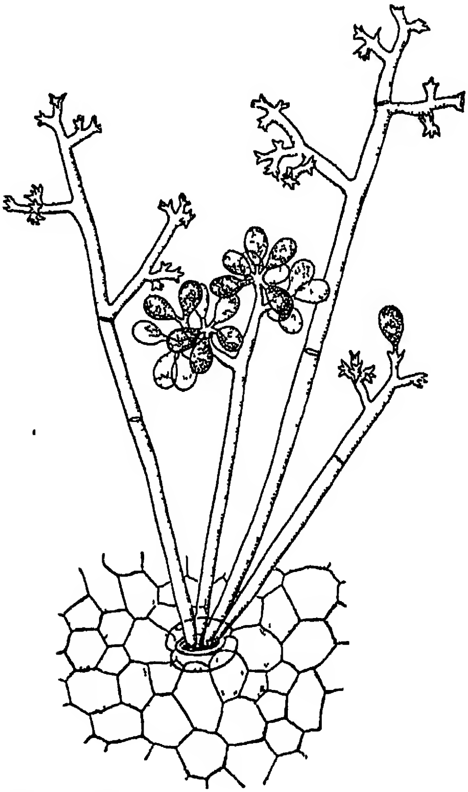


Fig. 47.—*Peronospora viticola* (after Cornu.)

ccording to the stage at which they are attacked; moreover, it estroys the foliage. The species is indigenous to North America, nd was introduced into Europe in the year 1878 along with American ines; it has now spread to all vine-growing countries. Vine owers are endeavouring to suppress this pernicious parasite by ie application of copper sulphate and calcium hydrate (Bordeaux ixture), and by similar remedies.

CHAPTER V.

YEASTS.

According to modern usage the word "yeast" is used to describe those alcohol-forming fungi which are normally formed by a process of budding Under special conditions many of them are able to form spores in the interior of the cells; the old name *Saccharomycetes* has been retained to describe these species, which are of such great technical importance

It has already been stated that both the bacteria and mould-fungi include alcohol-forming species, while amongst the moulds certain bud-forming species also occur

Mycelium formation has been shown to exist in, not a few *Saccharomycetes*, and since an endogenous spore-formation also occurs in certain of the moulds, it would appear doubtfully correct to class the yeasts as an independent group of fungi. The direct observation of genetic connection between typical *Dematium*-like mould-fungi on the one hand, and *Saccharomycetes* on the other, makes it difficult to accept the earlier view. Such observations at all events prove that species exist which cannot be classed in an independent group. Doubtless future investigations will bring to light further instances of species which represent stages in the development of higher fungi.

The genera *Mycoderma* and *Torula*, which include no members exhibiting endogenous spore-formation, but include a number of species known only in the budding stage, will be dealt with in an appendix to this section.

The Nutrition of Yeasts.

Some account of the nutrition of fungi in general has already been given in the chapter on moulds. In the following paragraphs the special nutritive requirements of yeasts are reviewed.

By way of introduction, we give a summary of the more important constituents of the yeast cell.

The mineral matter of yeast usually represents 5–9 per cent. of the dry substance. The chief constituents are phosphoric acid (about 50 per cent) and potash (about 30 per cent) The numerous analyses (made by Béchamp, Belohoubek, Bull, Champion and Pellet, Liebig, Lintner, Mitscherlich) show, as may be expected, considerable

discrepancies, partly because the yeasts examined were of different species and condition, and partly because the yeast had been more or less freed from the secretions of the fermented liquid. The following are the extreme figures given by the different analysts —

K_2O,	23 33 to 39 5
Na_2O,	0 5 to 2 20
CaO,	1 0 to 7 58
MgO,	3 77 to 6·31
Fe_2O_3,	0 06 to 0 7
P_2O_5,	44 8 to 59·4
SO_3,	0 57 to 6·38
SiO_2,	0·92 to 1·88

The analysis of **pressed yeast,** possessing great strength and stability, gives, on an average, about 75 per cent water, 1·6 to 1·8 per cent nitrogen, 0·5 per cent potash, 0·1 per cent. magnesia, and 0 6 to 0·8 per cent phosphoric acid (P_2O_5)

Among the **carbohydrates** may be named the material of the cell-membrane, the so-called **hemicellulose** and **yeast gum,** and further, **glycogen,** which at certain stages of the life of the cell may form a considerable part of its contents (17 to 39 per cent.) Glycogen occurs in larger quantity when the nutrients are rich in carbohydrates, they are stored in this form as reserve stuff. In the absence of nutriment, the cell gradually uses up glycogen. Its presence in yeast was proved by Errera, Laurent and others showed that yeast may store considerable reserves.

The **albuminoids** of yeast amount to about two-thirds of the dry residue (30 to 75 per cent, corresponding to about 5 to 12 per cent N) The amount depends on the nutrition of the yeast, supply of oxygen, temperature, etc In a brewer's yeast containing approximately 8·5 per cent N, Stutzer found about 64 per cent as albumen, 10 per cent as peptones and amino-acids, and 26 per cent as nucleins.

Amongst the nucleins must be specially noted the nucleo-proteids, which are of great importance in the development of yeast They are specially to be found in the nucleus.

The **fats** in growing cells constitute 2 to 5 per cent. of the dry substance, but in old cells considerably more Their formation is favoured by access of oxygen and an ample supply of carbohydrates They act as reserve foodstuffs

As will be seen later the direct action of alcohol (or alcohol vapours) results in a considerable increase in the fat contents.

Finally, we must note the **vitamines** discovered by Chr. Funk (*Journ Physiol*, 45, 1912), their constitution does not yet appear to be known with certainty They are believed to occur extensively in plants, and to be of essential importance for the nutrition of plants and animals They were first detected in rice husks, after a diet of polished rice had been found to cause a specific disease (polyneuritis), which could be cured by the addition of an extract

of husks Funk first isolated the vitamines from these and later from yeast, which contains especially Vitamine "B," soluble in water, but not in alcohol or ether The presence of vitamines greatly enhances the food value of yeast. According to extensive researches by Nelson (*Journ Biol Chemistry*, 46, 1921), yeast is able to synthetise for itself the water-soluble vitamine, which, therefore, constitutes a true constituent of the cell *

The elements phosphorus, potassium, magnesium and sulphur have been enumerated by A. Mayer as indispensable for the nutrition of yeasts. His statement is based on the results of his analyses of yeast (not pure cultures) and of his nutritive experiments. If malt-wort or other nutrient medium contains too small a quantity of phosphoric acid, the defect may be remedied by adding potassium phosphate Potassium is an essential food element for yeast It is readily absorbed, both in the form of phosphate and sulphate Magnesium is an element of equal importance Sulphur can always be detected in yeast, and must, therefore, be regarded as essential to its metabolism Calcium, on the contrary, does not appear to be necessary for the propagation of yeast. This element plays an important part, however, in the fermentation process, for it has been shown by Seyffert that brewery yeast quickly degenerates in a wort poor in lime. According to the recent work of Delbruck, Lange, Henneberg, Hayduck, Seyffert, and others, calcium carbonate renders certain toxic bodies innocuous, which are present in the raw materials and are believed to be of an albuminoid character (see Chap i.) In the preparation of artificial nutritive solutions for yeast, these ingredients should be added in the form of salts, the total quantity not exceeding a gramme per litre †

* Yeast is such a rich foodstuff that it is extensively applied in industry to the manufacture of nutritive preparations. They are generally prepared by bringing about an autolysis and auto-fermentation in the yeast-mass, to which is added minute doses of sodium chloride, carbonate, etc A second method is to prevent auto-fermentation by addition of considerable quantities of common salt (5 to 10 per cent), when the mass is sufficiently dissolved, it is filtered, the filtrate is boiled, evaporated, and suitably flavoured Yeast is further used extensively to enrich fodder; dry yeast (Zymin) has been used to great advantage as a therapeutic agent (acne, boils, etc)

† Ad. Mayer utilised —

Acid potassium phosphate, KH_2PO_4,
Crystallised magnesium sulphate, $MgSO_4$, $7H_2O$,
Tribasic calcium phosphate, $Ca_3P_2O_8$,

decreasing in quantity in the order given.

Laurent's solution contains —

0·75 g potassium phosphate per litre.
5 0 g ammonium phosphate or sulphate
0·1 g magnesium sulphate
1 0 g tartaric acid

Hansen's artificial culture fluid contains —

88·5 g distilled water
0 2 g magnesium sulphate
0 3 g monopotassium phosphate
1·0 g peptone (Witte)
10 0 g saccharose

Molisch found that traces of iron have a favourable effect on propagation of pressed yeast Kossowicz showed that in this ject ferrous sulphate is preferable to ferric chloride [r]

Among **carbon** compounds, the **sugars** play the most important in the nutrition of yeast Pasteur, experimenting on saccharose, id yeast absorbed about 1 per cent of the sugar contained he fermenting liquid to build up its cells The amount of sugar umed, and consequently the increase of cells, depends on quantity and variety of nitrogenous matter supplied, and on general course of fermentation, which is known to be largely ienced by the supply of oxygen Part of the sugar absorbed ot assimilated, but stored up as reserve stuff in the form of ogen With regard to the assimilation of individual sugars, yeasts react differently, but there is no parallelism between entation and assimilation in this respect The first investions were made by Beijerinck, who found that a brewer's yeast a Pastorianus yeast assimilated maltose, dextrose, and sacose · that a kephir yeast assimilated dextrose, saccharose, and ise and, further, that *Sacch octosporus* assimilated dextrose maltose, but not saccharose Artari proved *Sacch. Zopfii* ilated dextrose and saccharose, but not maltose A comensive study of a large number of species was made by Lindner by Saito, who determined the extent to which each sugar is assited by the yeast The brewers' bottom-yeasts examined were d to give a vigorous development with maltose, whereas dextrose assimilated less readily and saccharose not at all ; top-yeasts ed a weaker development with maltose, and frequently no lopment with dextrose and saccharose , most of the wine yeasts lly assimilated maltose, but dextrose less efficiently, and iarose hardly at all , most of the wild yeasts assimilated ose efficiently, while dextrose and saccharose were generally ilated more feebly but to a varying degree In a few species ht assimilation of lactose could be observed The two authors asparagine as the source of nitrogen, and remark that other es of nitrogen may be found to give quite other results.

r-grown yeast factories use inorganic compounds to advantage, particularly of nitrogen and phosphorus, to enrich the nutrient liquid, to regulate the coma of the medium and make good any deficiency of assimilable substances in molasses, etc It has been shown that the yeast-mass is most active and stable sence of 1 6 to 1 8 per cent nitrogen The proper use of such inorganic inds as are readily assimilated by yeast has led to increased yields of good yeast; they are, therefore, of particular value in factories concerned to produce but not alcohol Thus factories using molasses to produce yeast, add some 3 per f ammonium sulphate and a similar quantity of superphosphate, the former lirectly dissolved in water, but the phosphate repeatedly lixiviated with 50 times ght of cold water in a vessel fitted with a stirrer In factories aiming principally usively at the production of yeast, the yeast must be given the best facilities for g the nutriment to the best advantage To minimise the effect of alcohol, ment is carried on in a highly diluted wort, with a continuous supply of fresh e solution while fermentation is progressing

According to their experiments, maltose appears to be particularly well adapted for assimilation by yeast, but this result is probably due to the fact that the maltose used was strongly contaminated by albuminoids Similar research made at a later date Klöcker on the reaction of twelve races of yeasts on dextrose, lactose maltose, and saccharose He employed partly a mineral nutritive liquid (water 1,000 c c, magnesium sulphate 0 25 g, mono-potassium phosphate 5 g) with asparagine or peptone, and partly yeast water, the multiplication of the yeast was determined by counting the cells This thorough research proved that multiplication as well as assimilation of the sugars is largely dependent on the source of nitrogen Thus, *Sacch. Marxianus* in mineral solution plus asparagine was able to assimilate dextrose, maltose and saccharose, and even lactose by use of peptone. *S fragilis* in yeast water could assimilate dextrose, lactose, and saccharose, but in mineral solution plus asparagine none of the four sugars was assimilated Hence, under certain conditions, yeast can assimilate a sugar which it is unable to ferment The well-known species, *Sacch cerevisiæ, Carlsbergensis, ellipsoideus, Pastorianus*, and *turbidans* in yeast water and asparagine solution assimilated dextrose, maltose, and saccharose, but not lactose The value of asparagine as a source of nitrogen varied greatly with different species

Dextrins seem to be of doubtful value for the nutrition of yeast.

According to Laurent's researches, certain yeast-species are able to assimilate the alkaline acetates, and, further, lactic, succinic, gluconic, malic, tartaric and citric acids and their alkaline and calcium salts, in 1 per cent solution; likewise, glycerine, dextrin, and mannite in the same dilution. Will found certain yeast species actually capable of assimilating ethyl and amyl acetates

The importance of **alcohol** as a foodstuff for yeast was alluded to in the preceding chapter By parallel experiments with mineral nutriment and definite quantities of sugar and alcohol, in equal parts by weight, Lindner proved that the higher yield was obtained when alcohol was used as nutriment The assimilation of alcohol seems to be of consequence in modern processes of air-grown yeast manufacture, where fermentation is carried out in very dilute wort.

With regard to possible sources of **nitrogen** for yeast, it may first be noted that amongst *inorganic* sources, ammonium salts are readily absorbed. It was proved by Pasteur that yeast can grow in a nutritive fluid containing no organic nitrogen, but only nitrogen in the form of ammonium tartrate (100 c c. aqua, 10 grams sugar, 0·1 gram ammonium tartrate, and the ash from 1 gram of dried yeast) A Mayer obtained a positive result by the use of amm nitrate Willdiers subsequently proved by exhaustive experiment that it is not sufficient to supply nitrogen in inorganic compounds; Kossowicz reached the same conclusion, and showed

that by sowing a single cell in a saccharine fluid containing mineral matter no development took place, whilst with a greater inoculation of cells development may proceed

It has, however, been definitely proved in the case of *Mycoderma* species that their demand for nitrogen can be fully satisfied by ammonium salts

The yeasts can absorb the nitrogenous compounds of the **air,** as proved by Will, Kossowicz, Lipman, and others It is doubtful, however, whether they are able to assimilate pure nitrogen

According to the extensive researches of Ehrlich and Thomas, only such **organic nitrogenous** compounds are adapted to the nutrition of yeast as can readily and completely supply their nitrogen to yeast in the form of **ammonia,** which is of great importance for building up plasma-albumen in conjunction with the carbonaceous constituents of the nutriment Of particular value,, therefore, are the degradation products of the albuminoids, such as albumoses, peptones, amino-acids and their amides, asparagine and glutamines These substances either pre-exist in the raw materials used in industry, or they are formed in the course of the mashing and fermentation processes Thus, for instance, yeast converts asparagine into proteids. Certain decomposition products of proteids are already formed in the malt by the action of proteolytic enzymes Among the cereals, rye is particularly rich in proteids capable of building up the yeast-cell As to the quantities absorbed of these supplies of nitrogen, the yeasts show marked differences , thus some of them prefer peptone or yeast decoction to asparagine Ehrlich maintains that the amount of nitrogen absorbed by yeast is in direct ratio to the amount of sugar fermented.

Only part of the nitrogen contained in brewers' and distillers' wort is absorbed by yeast , grape-must likewise contains an excess. Musts of apple and pear are deficient in these compounds, and consequently are incompletely fermented, unless the deficiency of nitrogen is made up by an addition of ammonium chloride (Müller-Thurgau suggests 20 g per hectolitre), or by ammonium tartrate.

The importance of assimilable nitrogen to yeast life is considered by recent investigators to be twofold, peptones and bases being of particular value for the multiplication, whilst amino acids are found to induce the formation of zymase, and consequently the fermentative activity of yeast just as phosphates are necessary for the nutrition of yeast, on the one hand, and, on the other, play a prominent part in the very process of fermentation, as will be discussed later.

In the composition of the nutritive liquid, the ratio of nitrogenous compounds to carbohydrates is of essential importance for the normal growth and activity of the yeast, the fact being, that the cell, by thus fermenting sugar, creates energy which constitutes the fundamental source of the many chemical changes

which, consequent upon the utilisation of nitrogenous compounds take place in the growing cell. On the completion of fermentation, about one-third of the nitrogen contained in the liquid will have been assimilated by the yeast, independent of the concentration of the liquid It appears to be an established fact that, at any rate in the presence of certain nitrogenous compounds, such as peptone, fermentation will go on the more slowly, the higher the concentration of the compounds in the liquid, but, at the same time, growth and propagation will be more active When the liquor contains a larger proportion of albuminoids, more will be found in the yeast One and the same yeast, with varying content of albuminoids, will vary in fermentative activity

The growth and propagation of the yeast cell, which stand in an intimate reciprocal relation to the fermentative activity, are dependent not solely on nutrition, but also on the **direct action of external factors.**

As an example of a direct observation of the development and multiplication of the cell under definite conditions, Slator's research may be adduced. With the aid of a hollow slide, in which water at a constant temperature was circulating, the development of a single cell (bakers' yeast) was observed at 30° C in malt wort having a specific gravity of 1·04 A young cell developing freely was seen to put forth a bud which reached the size of the mother-cell in 73 minutes (the length of time varying 10 per cent. either side of the average, according to the condition of the cell) . within the next half-hour *no appreciable change* could be noticed , both cells then put forth simultaneously a bud, and the process was repeated. When old cells were introduced into a fresh medium, they remained quiescent for a time dependent on their age, and afterwards developed as stated above Under the conditions described, the retarding influence of carbon dioxide was directly observed to assert itself gradually Researches of this kind on low-fermentation yeasts in wort were made by Zikes, who found, in determining the influence exerted by the concentration of the wort on the development of the cell, that a single-cell culture of a Frohberg yeast in a moist chamber at 22°-23°-26° C has a somewhat higher velocity of growth during the first few hours in 1–5 per cent. worts than it has in 9–13 per cent worts

As regards the influence of **oxygen** on the development of yeast, it was noticed by A J Brown and, later, by H T Brown, Slator, and others, that the oxygen dissolved in the wort is very soon consumed by the yeast The Browns, as well as Pasteur, took the view that, with sufficient nutrition, free oxygen acts as a stimulant to the growth of yeast, as a *primus movens* in its future functions. H. T. Brown held that the growth of yeast is also influenced by oxygen adsorbed by the sown yeast By this conception, free oxygen is of direct importance to the further growth of yeast.

Slator, on the contrary, concludes from his experimental results that, at the stage when yeast is developing in wort, the *direct* importance of oxygen is much less than is generally supposed, and that its influence consists chiefly in preventing the air becoming saturated with carbon dioxide, the retarding action of which is much greater than had been supposed. In later stages, however, and under special circumstances, free oxygen may be of direct importance. Slator cites two instances of different modes of yeast growth, each involving a different set of chemical reactions.—(1) The initial stages of growth in malt wort illustrate one mode of growth, where oxygen, either free or combined, or adsorbed by the yeast, plays no part in the process, and free oxygen slightly retards it. The energy necessary for growth is obtained by fermentation of sugar, growth being retarded by carbon dioxide, for if air is bubbled through the fermenting wort, growth is accelerated owing to displacement of carbon dioxide. (2) The second mode of growth is illustrated by that occurring in lactose-yeast water, where there is no alcoholic fermentation, although zymase is present in the cells; free oxygen is essential in such a case, and, if absent, hardly any growth occurs, the necessary energy being obtained by an oxidation promoted by the yeast. A high percentage of carbon dioxide retards the growth. The factors determining which mode of growth will take place, when both are possible, have not been completely investigated. Hence as far as our present knowledge goes, it would seem that free oxygen is by no means always necessary for the vegetative growth and functions of the cell, but only under unfavourable conditions, such as those referred to, or when the nutritive fluid is deficient in suitable nitrogenous matter.

Spore formation in the cell cannot take place in the absence of ree oxygen.

As shown by Hansen as early as 1879, the multiplication of he cells is greatly accelerated by bubbling air through the nutritive iquid, fermentation being accelerated at the same time, the more o because, under such circumstances, the fermentative action of he cell will overcome any influences that tend to check fermenta-on. The physiological effect of a copious oxidation or respiration as demonstrated by Giltay and Aberson, by parallel cultures, ith and without aëration, in a 6 per cent. dextrose solution with-ut nutritive salts. More sugar was used in the aerated cultures than those not supplied with air, but the amount of sugar split up ito alcohol and carbon dioxide was smaller in the aërated cultures proportion to the yeast supplied. Thus, with an air supply, the east used up a larger quantity of sugar for its vegetative growth, e oxidation or respiration being freer.

As is well known this experimental fact is extensively used the manufacture of air-grown yeast, where the yeast is

treated in such a way as to utilise the largest possible quantity of nutrients for the multiplication of the cells, while the wort employed is excessively dilute and continuously renewed throughout the process

In this connection we may note that Hayduck proved that a strong oxidation of quiescent yeast renders it more resistant to long preservation.

In regard to the interference of external factors, it is hardly possible to lay down any general rule, as all depends on the condition of the cell and the composition of the nutrient medium. As regards **alcohol,** growth is influenced even by 2 per cent, and is completely arrested in presence of 7-10 per cent Muller-Thurgau found the temperature has a material influence on the effect of alcohol upon the fermentative activity of the cell, thus, 4 per cent. will arrest fermentation at a temperature of 36° C, whereas at 9° C. the same effect is only brought about by 9 5 per cent of alcohol. The influence of **carbon dioxide** has been discussed, it has, without doubt, a powerful effect on the condition and growth of the cell. In air-grown yeast manufacture, carbon dioxide is removed by a copious current of air through the liquor

As for the influence of **acids** on the development of the yeast and the course of fermentation, a certain amount of evidence, of great practical importance, has been procured through the recent dissociation theory (hydrogen-ion concentration) This evidence again proves that the nature of the nutrient medium plays an important part in determining the degree of action, and, further, that the different species or races react differently in presence of the same acid. It may probably be laid down as a general rule that the growth of the cell is more sensitive to acids than is fermentation, there is no parallel action of O and H ions on the two functions. The yeasts, in general, prefer a slightly acid medium, in contrast with bacteria, most of which prefer alkaline, and with moulds which thrive best on a more acid medium. The influence of acids on the propagation of yeast is illustrated by some observations of Hägglund —Under given conditions the yeast, with a concentration of 0·015 **hydrochloric acid,** had multiplied by 108 3 per cent in 120 hours, to compare with 59·2 per cent. without hydrochloric acid, with 0·024 per cent HCl, by 113 7 per cent, with 0 048 per cent., by 72·1 per cent The amount of alcohol differed slightly in the three cases. With **butyric acid** the following results were determined —Concentration, 0 005 · increase, 24 per cent, to compare with 57·3 without butyric acid, at 0 023, no increase **Lactic acid,** concentration, 0·111, increase, 89 per cent, to compare with 58·3 per cent without lactic acid, at 0·222, increase, 40·2 per cent It will be seen that, in these as in other cases, butyric acid powerfully inhibits the growth of yeast, whereas it is favoured, under the experimental conditions described, by

much higher concentrations of lactic acid As for the influence of **sulphuric acid** on yeast, it was shown by Henneberg that the power of resistance of yeast is largely affected by its actual condition Experimenting on pressed yeast, he found that, the richer in albuminoid matter the more sensitive it is, and that quiescent yeast could withstand 9 per cent sulphuric acid for fifteen minutes and up to 5 per cent for eighty minutes

Sulphurous acid, which even in minute doses will kill bacteria (*e g*, in wine-must), appears, according to Muller-Thurgau, to react very differently on species of true wine-yeast, on *Mycoderma*, *apiculatus* and *Torula* occurring in the must, some of them being quickly suppressed, while others, and among them several true wine-yeasts, will even survive severe treatment Hagglund found that the fermentation of 1 g of distillers' yeast in 25 c c of liquid was completely arrested by an amount of sulphurous acid corresponding to 0 007 n S It is, of course, impossible to lay down general rules as to the fermentative activity of the yeast cell, as the plasma contains a large number of enzymes, each of which, as shown experimentally, is sensitive to a specific acidity. In this respect also, the physiological effects of different acids doubtless vary The optimum hydrogen-ion concentration (P_{H}) for the action of brewers' and distillers' yeast is stated to range from about 4 4 to 4·7 On the contrary, the wild yeasts occurring in breweries were found by Emslander to have their optimum at a more alkaline reaction According to Geys, the acid reaction (P_{H}) of the wort in a yeast propagating machine declined with each new generation, when the yeast was left too long (two or three weeks) in the cylinder without being cooled down, at the same time the yeast lost its power of effecting clarification Rosenblatt's investigations, for details of which we must refer to his memoir, deal with the influence of a large number of acids and bases, and go to prove the correctness of the assumption that the extent to which they arrest fermentation is largely dependent upon the specific character of the cell.

Temperature is of capital importance to the development of the yeast-cell The significance of temperature with respect to fermentation in practice and to pasteurisation has been dealt with, so also the life limits In the systematic section will be found numerous instances of the significance of temperature in characterising the species and determining the form of the yeast-cell Temperature further determines the activities of the various enzymes of yeast, and in this respect also the respective groups and species react variously. Thus, in point of fermentative power, a comparison between a brewery bottom-fermentation yeast and a distillery top-fermentation yeast showed that they behave practically alike at temperatures below 30° C, but above this point the fermentative power of the top yeast was considerably

superior to that of the bottom yeast The upper limit of fermentation is, in general, about 50° C., while the lower limit of any species (and these few in number) is about 0° C. The formation of fat in the cells was shown by Zikes to take place very slowly at 12° to 15° C., but freely and in a comparatively short time at 20° to 30° C. Glycogen formation also proceeds at a considerably quicker rate at higher, than it does at lower temperatures, the optimum for distillers' yeast lying between 27° and 31° C (Henneberg), for brewers' yeast about 30° C. (Zikes).

As regards the influence of **light** on the functions of the cells, it has already been noted that, according to Kny and Lohmann, the budding process is not influenced by feeble illumination, but is retarded by diffused day-light, and that the effect of light is more apparent at higher temperatures Buchta, by observation of single cells, found that their sensitiveness to light is greater than was formerly believed Cells of *Sacch cerevisiæ* and *Sacch. Ludwigii* in diffuse day-light or electric light, multiplied only half as freely as in the dark Blue light tends to retard propagation, while in red light the cells were found to multiply with the same or with even greater velocity than in the dark As for ultra-violet light, Buchta observed that development was checked after ten seconds' exposure in a moist chamber, and that on prolonged exposure it was completely arrested in three minutes In aqueous dilution, yeast, according to Christoph, is killed in two to three minutes with a concentration of 2,000 to 3,000 cells per c.c., forming a layer not exceeding 5 mm, at a distance of 10 to 15 cm. In wort and beer the action is so slow as to be of no practical importance

According to accurate determination made by Luers and Christoph (1923), the velocity constant of annihilation may be taken as a practical measure of the radiation intensity, which is approximately proportional to the square of the distance The annihilation of the cells is retarded as the concentration of the suspension is increased The influence of temperature on the annihilation of yeast by radiation is very slight

As to the action of ultra-violet rays on fermentation, Romolo di Fazi (*Ann. di Chimica applicata*, II, vol. iv., 1915) instituted parallel fermentations in glucose with a brewery top-fermentation yeast in a quartz flask and a glass flask (through which the ultra-violet rays cannot penetrate), and found that notably greater amounts of carbon dioxide were produced in the quartz flasks. The distillate and the yeast in the quartz flasks were odourless, while in the glass they had a disagreeable odour Thus, the noxious germs (bacteria) proved unable to establish themselves in ultra-violet light

Similar results were obtained by Lindner in parallel fermentations of glucose with a brewery bottom-fermentation yeast, where

the rays impinged upon the bottom and sides of the flasks In six hours at about 26° C, six times, and after twenty-four hours, even 23 times as much carbon dioxide was formed in the quartz flask In quartz 20 to 30 per cent of the cells were found to be weakened or dead, whereas the other cells had a vigorous appearance The quantity of yeast had increased little, if at all.

It has already been noted that yeast will support very high **pressures.** According to Kilkwitz (*Ber d Deutsch. Botan. Ges*, 39, 1921), fermentation can proceed under a pressure of 40 atmospheres Even so the yeast was affected more by the narcotic influence of the carbon dioxide than by the pressure itself After subjecting to pressure for 3 to 5 hours, the older cells showed a contracted, the younger a homogeneous plasma If placed in nutrient liquid under normal pressure, the yeast continued the fermentation

The influence of **poisons** on the cell has been discussed in the first and third chapters The enfeebling or killing effect of a poison does not depend solely on its concentration, but also on the quantity which comes into contact with the yeast. An essential point in determining the toxic effect is, of course, the relative quantities of poison to yeast acted upon So also is the condition of the yeast, one and the same amount of poison may exert a widely different effect on yeast in fermentation and yeast in a state of repose Further, the influence of the poison on propagation must be distinguished from its reaction on the cell-enzymes This is illustrated by the contrast alluded to in the next chapter, between the powerful effect produced by toluene and chloroform on the plasma of the living cell and its effect on the free fraction of the alcoholic enzyme As already stated, temperature has some influence on the toxic effect. It is specially marked when the temperature is above the optimum of the specific yeast The stimulating effect of traces of poison was described in the first and fourth chapters We may specially refer to Bokorny's exhaustive researches on the action produced by various poisons on the yeast cell

Normal conditions of development of the yeast-cell exist, of course, only in nature In studying pure growths we endeavour to come as near as possible to the conditions obtaining in nature, we try to adapt the yeast to those artificial conditions most favourable to growth, the development then observed is described as normal If the factors or agencies described in this chapter are varied, the result will be more or less marked deviation from the ordinary course of development of the cell, **variations** come into existence, as described in some detail below, where some of those occurring in industry are also dealt with When yeast is subjected to external interference, it manifests a high degree of **adaptive faculty.** Instances of this are the adaptation to poisons (hydrofluoric acid, sulphurous

acid, etc) and the power of fermenting dextrin or galactose A few such variations appear to be hereditary. The properties or characters resulting from such adaptations and variations are usually those that the cell possesses under what are called normal conditions; they are merely brought to more marked development by special influences brought to bear upon them This adaptive faculty of yeast is of capital importance in its bearing on the fermentation industries

Theories of Fermentation.

It was long since observed that when a sugar solution or a fruit juice is exposed to air, fermentation phenomena occur after a lapse of time The liquid becomes turbid, gas is evolved, a precipitate is formed, and the surface is covered with a layer of yeast. The liquid gradually loses its sweet taste, at the same time clarifies, and then proves to contain a new substance with a stimulating action

What exactly the process might be was the subject of many speculations in olden time, but they were not based upon any true investigation of the process. We put all these speculations on one side, and start with the end of the eighteenth century, the epoch of the renowned Lavoisier, the "founder of modern chemistry," who gave the first explanation of the phenomena based on facts, the first hint of a theory of fermentation Simultaneously with the disappearance of sugar he proved that spirits of wine, carbon dioxide, and acetic acid were formed He explained the process as the splitting up of an oxide into substances both poorer and richer in oxygen. As the yeast played no part in determining the quantitative ratio of these he did not further concern himself with it

At the beginning of the nineteenth century Gay-Lussac published the well-known equation of fermentation, which still holds good, according to which a molecule of sugar was decomposed into two molecules of carbon dioxide and two molecules of alcohol The basis was thus given for a definition of fermentation—viz., a breaking down of complex bodies into bodies of simpler construction.

The question now arose in what way this transformation was brought about; what was the true cause of the decomposition of the liquid?

In the literature of the seventeenth, and still more in that of the eighteenth century, allusions are made to a "ferment" (Willis, Stahl) which was declared to be "a body existing in a state of internal motion" which transfers its motion to other bodies present in the liquid, whereby "the coupling of the compounds present is torn apart; the fragmentary particles are, however, through constant friction, attenuated and transformed into a new and more stable compound" These indications, however, remained

unheeded In 1810, Gay-Lussac, inspired by the brilliant chemical discoveries of Lavoisier, undertook to elucidate the process of fermentation, starting from Appert's method of preparation, which consisted essentially of preventing organic matter from undergoing fermentation by boiling it, and immediately afterwards sealing it tightly in vessels so that no air could penetrate to it. This process was, however, no new one, for as early as 1782 the Swedish scientist, Scheele, proved that acetic acid can be preserved unaltered after subjection to heat

Gay-Lussac examined the air contained in such hermetically sealed vessels, and found that it contained no oxygen. In his *Zeitalter des Sauerstoffs*, this observation led to the view that oxygen itself was the true cause of the process of fermentation, a view confirmed by the practical experience that sulphurous acid could be used for fuming out casks to arrest the fermentation of must, because the conversion of sulphurous acid into sulphuric acid brought about the removal of oxygen from the air of the casks.

The importance of yeast for the fermentation process was quite overlooked It was regarded as a precipitation from the liquid of no further importance for the comprehension of the process. The first indication of the true relationship had, however, been discovered at a much earlier period.

About the year 1600 two Dutchmen, Hans and Zacharias Janssen, invented the microscope, and in the latter half of the seventeenth century another Dutchman, Leeuwenhoek, issued his renowned letters on the investigation of various substances undertaken with the help of this instrument for the Royal Society. In 1680, in the course of one of his letters, a description and drawing of beer yeast appeared for the first time, and later in the same year one of wine yeast Shortly after, the first clear sketches of bacteria appeared He held the view that the yeasts were derived from the flour of cereals used in brewing, and he compared them with starch granules He had, however, no glimmering of the importance of yeast for fermentation

The observations of the learned Dutchman regarding the microscopic "animals," for so he named bacteria, originated the great discussion which extended into our time, involving researches and explanations of the important question whether these organisms can be derived from inorganic and dead matter (*generatio spontanea*), or whether they are derived from external fermenting and living matter All subsequent developments are based upon the investigation of this question, which naturally affected the domain of fermentation

After the fairy tales of earlier times had been disproved, Needham came forward in 1745 with definite experiments designed to show that the lowest microscopical forms of life, the "infusoria," were created in the following way.—He exposed decoctions of meat

and other organic substances to such a high temperature that, according to his view, all forms of living matter must be killed, and the vessels were then hermetically sealed When he opened them later he found living "infusoria" in the material, and he naturally argued that they were spontaneously generated, and that the substances generated during decay had recombined to form these microscopic germs of life

Needham's experiments were sharply criticised by Spallanzani in 1765, who proved, that if decoctions were maintained for three-quarters of an hour at the boiling point, no living forms were developed until air was admitted These experiments, incidentally, gave a rational basis to the processes of Scheele and Appert. Needham replied that this result could be explained in a perfectly natural way by assuming that the air present in the vessels was so altered in its character by continued heating that it was no longer able to maintain life Spallanzani was unable to combat this view experimentally, and so the matter remained undecided, and each view had its supporters The Needham school was upheld by the observation of Gay-Lussac that air, in hermetically-sealed vessels, contained no oxygen

No progress was made until the year 1836 From this time on began a period of rational investigation Franz Schulze proved for the first time that oxygen does not play the part that had previously been assumed, and his experiments also led to the introduction of the first indications of a biological theory alongside the dominant chemical theory Schulze vigorously boiled a mixture of water and organic matter in a glass flask, and then allowed air to pass through the flask after bubbling through sulphuric acid. This was carried on daily for a long time The result showed that the contents of the flask could be preserved unaltered for months together, whereas living forms of matter appeared in the decoction as soon as the vessels were opened and exposed to access of air.

At the same time Schwann carried out a similar experiment, with the exception that air was passed through a red-hot tube into the decoction, he obtained identical results

But, although these experiments proved that the air present in the flasks contained oxygen, and living matter was not generated, they proved unconvincing to the supporters of spontaneous generation They fell back on the theory that the powerful treatment the air had undergone had so altered its composition that it was no longer able to produce life

Schroeder and Dusch took up the subject in 1853-1861, with the object of proving that air containing all its gaseous constituents, unaltered, may be allowed to react on boiled fermentable material without effect It is unnecessary to expose the air to any vigorous chemical treatment with strong reagents, if it is first separated as far as possible from solid particles For this purpose

they made use of a filter of cotton wool, through which the air was led before it came into contact with boiled organic decoctions. Boiled meat and meat-broth, as well as malt-wort, were unaltered when filtered air was introduced into the flasks On the other hand, the experiments did not succeed with milk or the yolk of egg stirred up in water and boiled A completely decisive proof could not be furnished by Schroeder until, in 1861, he succeeded in sterilising this substance About this time Pasteur had begun a number of his epoch-making researches, in which the principle of sterilisation was clearly established

Prior to these, Schroeder presumed that in certain cases the air before filtration must contain something that could bring about fermentation and decay Whether these are "floating, microscopic, organised germs in the air, or a chemical substance, as yet unknown, which is separated by contact action and fixed on the cotton wool, must remain to be determined" It also appeared to be probable, in view of his negative experiments "that lower infusorial ferments exist, produced and separated either from living plant cells or from living animal tissue, which are capable of exercising certain organic functions and transformations." Mention must also be made of the experiments begun by Hoffmann in 1860 on decay and fermentation. He boiled the organic matter in a flask with a long drawn-out neck, bent several inches at an acute angle The subsequent inflow of air during cooling, deposited dust by gravity so that none could fall into the liquid.* The result was exactly that obtained by filtration; the liquid remained unaltered Notwithstanding all these observations, the school of spontaneous generation maintained their belief, and still numbered many adherents

In 1857, Pasteur, the distinguished French scientist, entered the field He submitted the problem to such conclusive experimental treatment, from every side, that his conclusions were generally adopted, and hold good to this day. He proved that the many unsuccessful experiments designed to overthrow the doctrine of spontaneous generation were occasioned by the fact that the organic liquids concerned had not been exposed to a sufficiently high temperature, or heated for a sufficiently long period Moreover, he showed that in such cases the liquid under treatment was *not* so greatly altered that it was no longer fit for the development of the germs, as supporters of spontaneous generation maintained Thus, if the liquid is boiled in a flask, the neck of which is drawn out to a tube and bent twice (the same idea as that of Hoffmann and Chevreul), so that the liquid remains sterile, and if a small portion of the liquid is then allowed to run into the tube, it soon begins to ferment, owing to germs deposited in the tube coming in contact with the liquid The same thing

* A similar arrangement was adopted by Chevreul.

occurred when air is passed through cotton-wool and a small quantity of the wool is introduced into the sterilised liquid. Pasteur also employed gun-cotton in place of ordinary cotton-wool Air passed through gun-cotton was sterilised, and the fluid, after boiling sufficiently, remained sterile for an unlimited time. The gun-cotton was afterwards dissolved in alcohol and ether, and it was proved that it contained the same microscopical organisms that develop in liquids undergoing fermentation and putrefaction

This great work of Pasteur's resulted in the overthrow of all proofs previously adduced on behalf of the school that maintained the spontaneous generation of microscopical life in organic liquids. He established an extremely important result for industry, which embodied all essential principles of the technique of sterilisation. The technique has now reached a high stage of development both in its purely scientific and practical aspects

Thus was laid the foundation of the belief that fermentation is brought about by living matter, the vitalistic theory of fermentation, in contrast to the chemical theory which found its best-known advocate in the distinguished Liebig, who built largely on the theories propounded by Willis and Stahl after Gay-Lussac's idea, that oxygen was the direct cause, had been given up.

Although Liebig's theory has been abandoned, it is necessary, even in a brief historical description to touch upon it, because it held the field for a long time, on account of its author's great renown, and has now been partially justified by the discovery of enzymes in the yeast-cell

Experimental chemistry had won great triumphs in the first twenty years of the nineteenth century Chemists had succeeded in ascribing extremely complex organic processes, previously ascribed to the mysterious "vital energy," to the simple agency of chemical affinities. As an obvious consequence, the attempt was made to explain fermentation phenomena in the same way, without the help of living beings Liebig consequently regarded the yeast which appeared in the fermenting liquid as a substance constantly undergoing decomposition, by which the chemical motion incidental to these processes was transmitted to the sugar, and brought about the decomposition of the latter It will be seen at once that this theory could not be held, when the presence of living and vigorous yeast cells was recognised Liebig, however, did not regard yeast as a plant, it represented to the chemist a substance without life, and microscopical investigation, according to Liebig, could contribute nothing of importance to an understanding of the process.

We shall now proceed to discuss how the knowledge of yeast developed and led to the vitalistic theory.

The Austrian Plenciz declared, as early as 1762, that decay

only takes place in a body when "germs of a wormy character develop and begin to multiply" Probably we have here the first definite announcement regarding the true cause of such decompositions

A long time elapsed before Leeuwenhoek's observations on yeast cells, in 1680, were carried a step further As far as we can judge from the known literature, the Austrian Erxleben in 1818 may have been the first who definitely expressed the thought that fermentation 'appears in no way to be a simple chemical operation, but rather is in part a process of growth, and should be regarded as the link in the long chain of nature which combines those actions that we describe as chemical processes with those of vegetative growth" But this must be regarded only as a hypothesis without further foundation

Twenty years later, and almost simultaneously, three scientists expressed clear and definite views, based on direct experiments, regarding the dependence of alcoholic fermentation upon yeast cells

It may be of interest to see how they arrived at the same result in three different ways

Cagniard-Latour was the first of the three to publish his work on yeast, in 1835-37 In his studies of beer and wine fermentation, both in practice and on the small scale, he observed that the yeast globules rise to the surface of the beer-wort on account of the entangled gas which they produce They possess the power, by budding or by elongating their own tissues, of multiplying and in this way producing manifold globules, which separate from each other when fully grown He thus confirmed his view that yeast cells are organic and belong to the vegetable kingdom. During propagation they are nourished by the beer-wort, and when the fermentation has come to an end the liquid contains many times the quantity of yeast that was added to it, whereas the earlier view was that the precipitate consisted mainly of secretions. He also found that yeast will not propagate in pure sugar solutions.

His researches enabled him to conclude that in all probability it is the yeast cells that destroy the stability of the components of sugar, and bring about its decomposition into alcohol and carbon dioxide. that fermentation, in fact, is a result of vegetable activity.

The same observation regarding the vegetable character of yeast was made simultaneously, or a short time after and quite independently, by Theodor Schwann It has already been mentioned that he made important contributions to the discussion of the generation of living matter, and it was these investigations that brought about his exact study of yeast under the microscope in 1837-39. Schwann arrived at the result that it is not atmospheric oxygen, but a substance conveyed by air, and destroyed by heat, which brings about fermentation To determine whether

this substance is of animal or vegetable character, he enquired whether it is destroyed by those poisons that are capable of killing infusoria, or by those that kill moulds. The latter proved to be the case, for a solution of potassium arsenite arrested the fermentation of wine, therefore, he argued, the substance must be of a vegetable character

Under the microscope, the yeast resolved itself into the recognised granules which constitute the ferment." He then observed how they form continuous rows, with other rows placed diagonally. He also observed that small granules appeared on the sides of the cells, which form the starting point for new rows, and usually, on the last granule of a row, appeared a tiny, and sometimes elongated body It will be seen that this constitutes an exact description of a budding colony of yeast resulting from direct observation under the microscope Schwann observed that the similarity between this picture and that of many other kinds of fungi was considerable, and this strengthened his belief that yeast is a plant. At his instigation Meyen examined "this substance," and gave the plant the name it has since retained of *Saccharomyces* (sugar-fungus)

Schwann also demonstrated that the feeble evolution of gas in grape juice may be regarded as a sign of fermentation. immediately afterwards the first individuals of the sugar-fungus made their appearance, these plants grew and multiplied throughout the period of fermentation. As it had also been shown that fermentation ceased through every treatment which brought about the destruction of the fungus (boiling, addition of potassium arsenite, etc), the connection between fermentation and the sugar-fungus could not be denied, and "it is extremely probable that the latter brings about the phenomena of fermentation through its growth" He declared that fermentation was carried out in such a way that "the sugar-fungus absorbs sugar and a nitrogenous body necessary for its nutrition and its growth, whereby those elements, which are not taken up by the vegetable body, are principally combined to form alcohol (probably along with many other substances)"

F. T Kutzing was the third who dealt with this important problem at the same period (1834-37) Within the scope of his elaborate investigations concerning the lowest microscopical plants, he included the yeasts and other micro-organisms that usually occur in brewery wort and distillery mash, and published good drawings of the growths It is of particular interest that Kutzing was the first to investigate "mother of vinegar," the slimy skin which forms on the surface of a liquid that is undergoing acetic fermentation. He examined this film from its earliest stage, and found that it consisted of very small plants, which gradually increase in length He realised the extraordinary importance which the study of the lowest forms of life would have for organic chemistry

and for the whole field of natural science Chemistry must rule out yeast from amongst its chemical compounds, as it proves to be an organism, and he regarded it as certain that "the whole process of spirituous fermentation is dependent on the formation of yeast, and that of acid fermentation on the formation of "mother of vinegar", "fermentation is synonymous with the vital process" Thus he supplied a clear and definite form for the vitalistic theory of fermentation, in opposition to Gay-Lussac's oxygen hypothesis, and to Liebig's theory of the breaking down of yeast cells as the cause of fermentation

Mitscherlich's work is also of a fundamental character In 1841 he described yeast as consisting of round and oval globules, and he solved the question of their importance for fermentation by the following beautiful experiment:—A little yeast is placed in a glass tube, closed at the lower end by a sheet of paper and this is placed in a sugar solution In the course of several days, it will be seen that fermentation has actually taken place in the tube, owing to the sugar solution having diffused through the paper Alcohol gradually diffuses throughout the liquid, which becomes saturated with carbon dioxide, but the greater quantity of carbon dioxide is evolved It is only after some time, when the paper softens and allows the yeast globules to pass through, that the fermentation process begins to take place on the surface of the paper He concludes that "fermentation only takes place at the surface of the globules" He also published beautiful drawings of yeast, showing their methods of growth and propagation and described the contents of the cell after staining with iodine

All these observations did not suffice to establish the new theory The great prestige of the chemist, which still prevailed, demanded an equal authority in the region of biology to take up every point of the discussion and by demonstrating the defects still inherent to the experiments just described and by convincing experiments along the whole line, to compel attention

This great work was carried out by Pasteur with the same conclusive results as in the case of generation The investigations begun by Pasteur did not consist, like those of the earlier experimenters, of short, isolated bits of work, but ranged over a series of years from 1857 onwards, and were published in a number of memoirs In this short review it is impossible to do more than indicate a few isolated and especially important experiments taken from the series, which ranges over the whole field of fermentation

At an early stage he made the important observation that the amount of sugar decomposed during fermentation is greater than that corresponding to the carbon dioxide and alcohol produced. The remainder of the sugar that disappears is utilised by

the yeast during fermentation, partly for its propagation—a fact which cannot be reconciled with Liebig's view, for he demanded, as a condition of fermentation, that yeast should be in a state of decomposition Shortly after, Pasteur proved that during fermentation, yeast not only produces alcohol and carbon dioxide, but simultaneously, succinic acid and glycerine, the latter derived from a further part of the sugar decomposed He also showed that by the addition of ammonium tartrate to the fermenting liquid yeast can be brought to more rapid development, and the liquid can be more highly fermented than usual, proving that this salt must be a food-stuff for yeast In general, he proved that no decay of yeast takes place during fermentation, and that the presence of assimilable albuminoids in the liquor is unnecessary, for by sowing a minute portion of yeast in a liquid which only contains sugar, ammonium tartrate and a few salts, fermentation can be brought about with the development of young cells capable of propagation

At the same time, he showed that the reason why many of the early experiments, which should have refuted the older theories did not succeed was due to the fact that it was impossible at the time to secure absolute sterilisation of the liquids

He then produced further proofs that the acetic acid fermentation, already recognised by Kutzing as due to physiological activity, must clearly be regarded as having this character

One further observation must be mentioned on account of its wide-reaching importance He proved that calcium lactate can undergo fermentation resulting in the formation of butyric acid and that the active organism can exist without access of air. He gradually extended his observations in this entirely new field (**anaerobiosis** = life without air), and definitely distinguished between aerobic and anaerobic life It was this remarkable discovery, which at a later stage included the alcoholic yeasts, that led the distinguished scientist to a solution of the problem under what particular conditions yeast cells can decompose sugar

In 1876, in his *Études sur la bière*, he formulated his celebrated theory of fermentation, based upon a series of actual experiments, details of which cannot be given here, a theory which has served both as the basis and the starting point by which progress has been made throughout a long series of years, and one which will always retain its importance It starts essentially from the idea that living yeast cells under certain conditions are obliged to live apart from air, and that they then react as exciters of fermentation. Fermentation is, therefore, bound up with the life of yeast cells; it is life without air As yeast under these conditions is obliged to obtain its necessary demands of oxygen from sugar in order that it may continue to develop as a living organism, it splits up the sugar, and the residual oxygen and carbon constitute new

compounds—viz , the fermentation products alcohol, carbon dioxide, etc At the same time, Pasteur emphasised the idea that in each kind of fermentation—alcoholic, acetic, butyric, etc —a specific kind of organism occurs

It will be seen that Pasteur's theory has both a biological and a chemical aspect The yeast cells fulfil their normal existence with ample access of atmospheric oxygen , under these conditions develop, according to his view, most vigorously, and prepare themselves in the best possible way to continue their existence without air that this is the necessary condition for their existence as alcohol formers—i e , decomposers of sugar The first statement, which clearly explained an important biological problem, still holds good , the second, which endeavoured to furnish an answer to the requirements of the chemical process, can no longer be accepted

That Pasteur did not apply his definition in the narrowest sense of the word is shown in that he himself emphasised the fact that yeast can exercise fermentative power in presence of a limited supply of air, as well as in its absence This was established under certain conditions for low fermentation beer-yeasts by Pedersen in 1878, and Hansen in 1879 They discovered that the quantity of dry substance in beer-wort which a given quantity of yeast can convert into alcohol, carbon dioxide, etc , is smaller when the liquid is aerated during fermentation than when it is not A similar result was obtained by Eduard Buchner in 1885 in his experiments on bacteria.

Nageli, in 1879, in his *Theory of Fermentation*, proved that access of oxygen is always favourable to alcoholic fermentation in a sugar solution, if no nutritive material is present, and consequently the quantity of yeast is only slightly inc d Nageli says (p 26), "The theory of Pasteur, that ferm ion results from a lack of oxygen, forcing the yeast cells to secure their requirements of oxygen from the fermenting material, is opposed to all the facts brought to bear upon this subject "

This view is shared by A J Brown He arranged a set of experiments in which fermentation proceeded in presence of full access of oxygen, and a parallel set in which oxygen was excluded In both series the same number of yeast cells were used, and they were kept under such conditions that it was impossible for them to multiply otherwise every condition was the same It proved, contrary to Pasteur's theory, that the cells in the first case developed a higher fermentative activity than when oxygen was excluded

Similar experiments were undertaken by H Buchner and Rapp, with the object of ascertaining by exact quantitative methods to what extent free access of air brings about the replacement and suppression of the fermentative power of yeast cells by their oxidising function With this object in view, they prepared pure, surface cultures of yeast with the greatest possible access of air,

and carried out parallel experiments with limited access The first lot of cultures were grown in large cylindrical vessels, the inner wall of which was covered with a thin lining of wort-gelatine containing 10 per cent of grape-sugar This was infected with a coating of pure yeast, and in each experiment a current of air was passed through the vessel for five days The carbon dioxide was absorbed in caustic potash, and after each experiment the amount of alcohol and the ratio between the yeast and the fermented sugar were determined Parallel experiments were carried out, in which the same quantities of beer-wort and grape-sugar were allowed to ferment in Erlenmeyer flasks

As a consequence of the rapid and abundant growth of yeast on the surface of gelatine, fermentation on gelatine ceased much more quickly than in wort, where the yeast collected on the bottom of the flask It was further proved that considerably more carbon dioxide was formed in the surface cultures than in the parallel experiments with wort This carbon dioxide must be due to the respiration of the yeast. Nevertheless, only about one-seventh of the sugar was decomposed by oxidation, whilst more than six-sevenths were fermented Although yeast had been submitted, according to Pasteur's view, to the most favourable conditions for life without fermentation, nevertheless fermentation ceased As is now universally known, a free supply of oxygen exercises a favourable influence on the propagation of cells but these experiments served to establish the fact that oxygen has scarcely any influence on the process of fermentation, and that the absence of oxygen must not be regarded as being a condition of fermentative activity, for even in presence of a full quantity of oxygen the fermentative power of yeast still exceeds the respiratory power

From Nägeli's many-sided work on the lower organisms, we can only refer, in connection with the preceding, to his "molecular-physical" theory of fermentation, which may be regarded as a modification of Liebig's theory Whilst Pasteur regarded fermentation as the result of an activity taking place in the cell, Nägeli defined fermentation as a transference of states of motion of the molecules, groups of atoms, or atoms of different compounds constituting living protoplasm (which otherwise undergo no change) to the fermenting material, whereby the stability in these molecules is destroyed and disruption is brought about During fermentation the vibrations of the molecules of protoplasm are transferred to the fermenting material The cause of fermentation is to be sought in the living protoplasm in the interior of the cells, but its activity extends for some distance outside their walls The decomposition of sugar takes place to a slight extent inside the yeast cells, but principally outside them

We now return to the epoch-making researches of Pasteur.

He proved in the clearest and most unmistakable manner in his *Études sur la bière* what power is possessed by microscopic life, and he strongly emphasised the fact that bacteria may have a far-reaching influence on the course of alcoholic fermentation and on the character of the beer The budding organisms were dealt with in a similar way He indicated that certain fungi of this group, which are not described in detail, may react in different ways on the products of fermentation, as Bail had previously experienced Pasteur's communications, however, only traversed the nebulous views of his predecessors, and his assumptions led to two opposing lines of thought This is seen, for instance, in his observations on the so-called cheesy and aerobiotic yeast. It is possible that we have to do in this case with independent and peculiar types of yeast, but it is also possible that we are dealing with forms which are brought about by a particular treatment of the usual brewery yeast It should not, however, be overlooked that he himself indicated the direction in which the solution of the question must be sought, but that it was, at the time, impossible to determine whether one or more species was present; an exact method for the pure culture of yeast species not having been discovered Thus a true orientation in the world of micro-organisms cannot be found in his work It was impossible at any point in Pasteur's thesis to find characters described for the budding fungi that would enable an analysis to be based upon them He believed that all budding fungi may to some extent possess the power of bringing about alcoholic fermentation like the *Saccharomycetes*. It is never possible to tell whether he is referring to true *Saccharomycetes* or to other budding fungi Pasteur did not differentiate between the several kinds of budding fungi (*Saccharomycetes*, *Torula*, *Dematium*, etc)

Pasteur took the standpoint that every individual fermentation: lactic, butyric, acetic acid, etc , is produced by a particular exciter of fermentation It was only when the technique of pure cultivation had been further developed that an explanation of the true connection was possible—viz , that *each one* of these processes can be carried out by different kinds of organisms. This was proved by E C Hansen in 1878 for acetic acid bacteria, by Miquel for uric acid bacteria, and by Hansen in 1883 for the alcoholic yeasts.

The chief reason why the reform in brewing technique could not be carried out was that the current scientific standpoint made it impossible clearly to define the relations existing between the different yeasts concerned in alcoholic fermentation Pasteur was, therefore, unable to escape from the indistinct assumptions and the contradictory views of his predecessors In a review given in his book (pp 4-7) regarding the micro-organisms that bring about diseases in beer, he speaks only of bacteria, and his belief

is reiterated by Duclaux in 1883, and by other French, German, and English workers As a result of his studies, Pasteur recommended brewers to undertake the purification of their yeasts · to rid them of bacteria by cultivating them in a sugar solution with tartaric acid, or in wort, with a little carbolic acid

In contrast to all this, in 1883, Hansen published his doctrine that some of the most dangerous and most commonly occurring diseases in low-fermentation beer are not produced by bacteria, but by certain species of *Saccharomycetes*, and that the names *S cerevisiæ*, *S Pastorianus*, and *S ellipsoideus*, suggested by Reess, do not indicate one, but several species and races. Hansen proved that species which had been incorrectly grouped under the systematic name *S cerevisiæ* yield different products in the brewery From this standpoint he elaborated his system, utilising a stock yeast derived from a single species After some opposition the system was adopted in all brewing countries, and introduced to the industry Hansen's experimental demonstration showed that Pasteur's process for purifying yeast by means of tartaric acid, furthers the development of disease yeast to such an extent that they are capable of completely suppressing the true culture yeasts. Pasteur greeted Hansen's system as an advance, and wrote, "Hansen was the first to realise that beer yeast should be pure, and that, not only in regard to microbes and disease ferments in the narrower sense, but also that it should be free from cells of wild yeast"

The main problems regarding *the actual cause of the decomposition of the sugar molecule* and the *special conditions under which it takes place* still awaited solution

Meanwhile, in the last decade of the nineteenth century, new views regarding the fermentative forces were gaining ground, for it proved possible to separate the exciter of fermentation in certain cases (diastase from malt, pepsin from gastric juice) The characteristic effect of these ferments was that minute quantities were able to split up large amounts of the given material, and that they completely lost this power when subjected to heat The name **enzyme** was applied to the substances isolated from the living cells of the barley corn, the mucous membrane of the stomach, etc., and gradually a large number of these ferments were distinguished, amongst them some of great technical importance.

The thought naturally suggests itself that it must be possible to find an enzyme amongst the many elements of which the living yeast cell is constituted, capable of splitting up sugar As early as 1858 we find a suggestion of this kind put forward by Traube :—That "the chemical processes going on in living organisms originate mainly in the circumstance that protein substances are liable to undergo decomposition in the presence of water, and that under the peculiar conditions actually obtaining they are also

apt to give rise to peculiar ferments" A direct outcome of this view was Miquel's discovery in 1890 that the bacterium causing the ammoniacal fermentation of urine contained an enzyme which can bring about this fermentation on its own account.

In 1894 Emil Fischer, by purely chemical research, resulting in his celebrated work on the synthesis of the sugars, on the use of phenyl-hydrazin, and the osazone-reaction, diverted the current views on fermentation phenomena into new channels. His researches led him to explain the reaction of the yeast cell on the specific sugar of a nutritive liquid, in the same way as that of the enzymes (invertase, emulsin), so that the chemical activity of the living cell should not differ from the action of chemical ferments. According to Fischer, fermentation of polysaccharides is always preceded by hydrolysis of the sugar But there exists an exact relation between the molecular structure of a given sugar and the sugar-inverting enzyme of a yeast cell; if a sugar comes into contact with the albuminoids of a yeast cell, which are the outstanding agents utilised by the living cell, it is decomposed only if its configuration, the geometrical structure of its molecule, does not deviate substantially from the configuration of the albuminoid molecule Thus, according to Fischer's theory, the function of the living cell depends much more upon its molecular geometry than on the composition of the nutritive material.

Another way in which Fischer, as well as Thierfelder, obtained confirmation of his fermentation theory was by examining the reaction of Hansen's and other yeast species on the artificial sugar species, obtained synthetically by Fischer. They found, indeed, that the yeasts are quite fastidious regarding the geometrical configuration of the sugar molecule, whilst often they are unaffected by other alterations in its composition.

Among the various synthetically-prepared sugars tested by Fischer for their reaction on yeasts, **melibiose** is especially distinguished It is fermented by brewers' common bottom-fermentation yeasts, but not by many brewers' top-fermentation yeasts. In harmony with this, Fischer found that bottom-fermentation yeast contains an enzyme capable of extraction from the dried yeast in aqueous solution, which decomposes melibiose, converting it into glucose and galactose, but in a corresponding treatment of the brewers' top-fermentation yeasts no decomposition of this sugar could be observed As brewers' top-fermentation yeast contains invertase, it follows that the ferment which splits up melibiose cannot be identical with invertase

C. J. Lintner and Fischer showed, by methods devised by the latter, that natural maltose is split up into two molecules of hexose, if acted upon by an aqueous extract of dried yeast, or by cells, the membrane of which has been torn by grinding with powdered glass, and that there is a marked difference between this enzyme

and invertase, which hydrolyses cane-sugar The former enzyme is termed yeast-glycase or yeast-maltase. Its optimum temperature is about 40° C, whilst that of invertase, according to Kjeldahl, is 52°-53° C In a similar way, a lactose-cleaving enzyme (lactase) and an enzyme resembling invertase were isolated from *Monilia*.

At the same time, Hans and Eduard Buchner were endeavouring to prepare a juice by a treatment of yeast cells similar to that adopted by Emil Fischer—*i e*, by grinding the cell-wall hoping to apply it to therapeutic experiments To preserve the juice, it was mixed with sugar, and E Buchner observed that a vigorous development of gas took place in the mixture A further examination showed that the gas was carbon dioxide, and that alcohol was simultaneously produced in the juice This was the basis of the extensive research which led to the discovery of the alcohol enzyme, which was successfully isolated from the living cell (first communicated in 1897)

Buchner's process is as follows.—Freshly washed and highly pressed yeast is ground with quartz and infusorial earth in a mortar. The cells are torn and broken open by the sharp sand, and the juice absorbed by the infusorial earth In a few moments the whole mass cakes together to form a dough This is wrapped in strong press-cloth, and subjected to very high pressure in a hydraulic press, up to 90 kilogrammes per square centimetre From each kilogramme of yeast about 500 c c of clear yellow or yellowish-brown juice is obtained When the juice is mixed with a solution of saccharose, grape sugar or maltose, strong frothing takes place in a few minutes, due to the development of carbon dioxide, and at the same time almost the equivalent quantity of ethyl alcohol is produced By the addition of minute quantities of alkalies (potassium carbonate, disodium phosphate, etc), the process of fermentation is quickened

It can be shown that the fermentation is not caused by living cells remaining in the juice, for it is possible to add strong antiseptics like chloroform, thymol, or toluol,* which would arrest every living function of the cells, or again, the juice may be filtered free from germs through a porcelain filter, without destroying its activity It might be supposed that the fragments of protoplasm torn from the cells could be regarded as carriers of this power, and that the enzyme itself had not been isolated This cannot, however, be the case, for if the juice is treated with precipitants like alcohol-ether or acetone, the active substance is thrown down and this, along with other precipitated substances, on drying, forms an amorphous and very stable powder which, on treatment with water, can once more be employed as an exciter of fermentation

It has since been shown that high pressure is not essential

* On the other hand, mercuric chloride destroys the fermentative power of the juice.

R Albert has shown that by treatment of yeast with alcohol-ether, or, better still, with acetone in such a way that all the cells are destroyed, a very active powder can be prepared (**zymin**). The yeast is partially dried and soaked for a quarter of an hour in acetone (ten times its volume). It is then spread on filter paper to dry, washed with ether, and dried at 45° C The preparation takes the form of a white powder The powder, which consists of dead unbroken cells, produces almost immediate fermentation in a sugar solution If it is washed with water, the water does not acquire any fermentative power If, however, the cells are first disintegrated, it is possible by simple suction with a water pump to obtain a juice from which a precipitate is thrown down by means of ether and alcohol, which can be dissolved in water and immediately produces a vigorous fermentation in a sugar solution Whilst yeast that has been killed in the usual way does not retain any alcoholic enzyme, it is possible by this method to fix the enzyme so that it remains intact in the dead cells.

Buchner named this substance **zymase** (*Alcoholase*)

A Lebedeff showed in 1912, that zymase can be extracted from the yeast cell by simple maceration The procedure is the following —In a tank of at least 50 litres capacity, fresh yeast is washed by a continuous stream of water until the water flows away clear and the sediment is perfectly white Moisture is then expressed and the yeast spread out on filter-paper in a layer of 1 to $1\frac{1}{2}$ cm and allowed to dry for two days at 25°-35° C It is then macerated in water (50 g of dried yeast and 150 g water) at 35° C for two hours, or 25° C for six hours, and passed through an ordinary filter In 12 hours 70 to 80 c c of juice are obtained. The cells appear microscopically intact The juice will keep for a long time in the frozen state It is richer in extractive matter and more active than Buchner's juice It does not contain glycogen, but all the enzymes that are found in Buchner's juice

Thus we have a basis for the study of the specific enzyme which brings about fermentation A short *résumé* is given below of the properties of the enzyme It must, however, be understood that very little is known concerning its chemical character

If the juice is heated to 40°-50° C, a flocculent precipitate of albumen forms, and the clear liquid loses its fermentative power. nvertase has been identified in yeast-juice, and it must also contain an enzyme hydrolysing maltose and one hydrolysing glycogen, as t is capable of bringing about fermentation with these carbo-ydrates, but, according to Hahn, it also contains a substance f importance, a proteolytic enzyme hydrolysing albumen If a est tube containing fresh yeast-juice, and another containing uice that has stood for a week at room temperature in presence f toluol (to prevent the growth of micro-organisms), are placed n a water-bath at 40°-45° C., it will be found that in the former a

dense coagulum separates out in a few minutes, whilst in th latter only a few flocculent particles are visible The coagulabl albumen, when kept for some time, disappears by a species c auto-digestion Hahn has named the enzyme **yeast-endotryptas**e This enzyme reacts best in presence of acid, whilst the activit of zymase is improved by the addition of weak alkali The presenc of oxygen is advantageous to proteolysis The enzyme can b isolated in a comparatively pure state, and is found in yeast cell According to Hahn, it cannot be separated from quite norma cells

Endotryptase has a powerful action on zymase, and even whe the juice is kept at a low temperature a marked loss in its fermei tative power is observed in the course of a few days, owing t the action of endotryptase It is quite possible that it is th enzyme, more strongly developed, which attacks the enzyme (yeast cells when they are exposed to unfavourable condition Buchner believes that this accounts for the fact that yeast-jui prepared from one and the same species of yeast may contain ve variable quantities of zymase Zymase is extraordinarily sensiti both to variations in temperature and to the presence of stro alkalies To protect the juice from the action of endotryptas large additions of cane-sugar have been employed Thus, wh mixed with 75 per cent sugar solution, the activity of the enzyn has been prolonged for several weeks.

To avoid the rapid decomposition of the juice, it may be dri in a vacuum at 25°-35° C It forms a yellowish powder, whi remains unchanged for a long time and, when dissolved in wat displays almost undiminished fermentative power

As a result of a number of fermentations, Buchner notes th the fermentative power of 20 c c of yeast-juice, with the additi of 8 grammes of sugar and 0 2 c.c of toluol, at a temperature 22° C, gives a yield of 1 87 grammes of carbon dioxide

Compared with the fermentative power of fresh yeast, the activi of the juice appears trifling Thus, 1 gramme of good press yeast produces, in an 8 per cent cane-sugar solution, 1·5 gramn of carbon dioxide in six hours at 30° C, whilst 20 c c. of yea juice is produced from about 40 grammes of yeast

Thus, the fermentation produced by the living cell consideral surpasses that set up by free zymase. On addition of antisept (toluene, ether, chloroform) free zymase is but slightly attack whereas the fermentation induced by fresh yeast is arrested reduced to a minimum. These facts can only be accounted by Pasteur's theory, that the alcoholic enzyme of the cell, wh is formed by living plasma, is chiefly combined with the lat as a chemical complex, which is accordingly rendered inact when the vital action of the cell is destroyed, a relatively sn fraction might possibly be liberated by the plasma of the living c

or only after special treatment of the cell Such treatment may, however modify the constitution of the liberated enzyme, as would appear likely from the well-known fact that neither press juice nor acetone yeast, nor maceration juice, is able to ferment dilute solutions (e g, 10 per cent grape-sugar solution), whilst it can ferment concentrated solutions, such as 20 to 40 per cent. cane-sugar solution This has been the subject of exact research by Abderhalden, Armstrong, Euler, Giaja, Hayduck, and others

In his experiments on the nutrition of yeast, Rubner arrived at the same result by measuring the rise of temperature caused by fermentation The yeast does not develop more heat than can be accounted for by the decomposition of the sugar. Seeing, however, that a change of energy must take place in the cell, the vital energy of which is supplied by the fermentation, it follows that the decomposition of the sugar cannot be simply of a fermentative nature, but that it is necessary to distinguish between a "fermentation action" and a "vital" fermentative action

With regard to the best conditions of temperature, it has been shown that the highest fermentative activity of zymase is reached at 12°-14° C The most favourable temperature undoubtedly lies higher, but it must be remembered that at the higher temperature endotryptase immediately comes into action and attacks zymase.

The living yeast cell contains varying quantities of free zymase. Thus the content often increases perceptibly in quiescent pressed yeast when kept at low temperatures It is a remarkable fact that yeast cultivated in a strong sugar solution with inorganic salts secretes a comparatively small amount of zymase at the moment of greatest fermentative activity accompanied by the greatest production of froth. If, however, the yeast is removed at this stage, washed, pressed and stored for a few hours at a low temperature, it will be found that the zymase content has considerably increased. In the same way, yeast taken fresh from the brewery sometimes shows an increase of zymase after storing. These facts can be explained on the assumption that endotryptase is influenced by a low temperature, even when the other conditions are favourable.

On the assumption that under certain conditions it would be possible to induce yeast to meet its energy requirements, not by respiration but by zymase fermentation, Hayduck and Haehn undertook experiments with a *Torula* species which was found to multiply freely with a good air supply, whilst producing a very feeble fermentation. On cultivation in a nutrient liquid composed of molasses and salts, without access of air, a growth was obtained in a few generations which, in parallel experiments, was found to possess about the same fermentative power as distillers' yeast. This increased effect proved to be mainly due to zymase combined

with the plasma, as the dry preparation (zymin) did not produce any fermentation. Thus, as in the case of common distillers' yeast, no free zymase was found in the *Torula*-yeast

A short review of the chemical changes that take place during the fermentation of yeast-juice follows It is to be premised that glucose, fructose, maltose, and saccharose are fermented approximately with the same rapidity

The first problem is to discover whether the phenomena caused by the addition of yeast-juice to sugar solutions are identical with those of alcoholic fermentation of sugar The chemical action of the enzymes already discussed—invertase, maltase, lactase, diastase—consists in the hydrolysis of the polysaccharides into simpler compounds, the monosaccharides Zymase is distinguished from the enzymes by bringing about the complete break-down of the sugar molecule and the formation of new compounds, exactly like the alcohol enzyme of the living yeast cell. As is well known, this splits up sugar into almost equal parts of alcohol and carbon dioxide This is also the case with zymase A portion of the sugar, however, is not converted into those products.

During the pressed-juice fermentation, glycerine is produced to the extent of from 3 to 8 per cent of the fermented sugar; it is derived from the sugar On the other hand, no succinic acid is produced Acetic acid is formed in minute quantities, but somewhat more than in the fermentation with the living cell. This is probably due to the action of a special enzyme

The living cell does not apparently produce lactic acid in the fermentation On the contrary, this acid is formed in cell-free fermentation.*

Buchner and Meisenheimer have obtained a pressed juice and also a stable preparation from yeasts fermenting lactose, which are capable of carrying on fermentation.

Besides the enzymes already named, yeast-juice contains an oxidising, a reducing, a fat-decomposing enzyme, one splitting up hydrogen peroxide, and a rennet enzyme.

It has not yet proved possible to isolate zymase, and nothing is yet known regarding its composition Its properties may be summarised as follows.—It is soluble in water and dilute glycerine, and is not very sensitive to chemical reagents In solution it is decomposed at 60° C When yeast juice is kept at low temperatures (down to 0° C.), the zymase gradually disappears, whilst in a frozen condition it remains unaltered for some time. In a dry condition it may be stored for months with unaltered activity,

* It was recently proved by Fernbach and Schoen that in the course of common alcoholic fermentation some lactic acid is produced, which, however, is destroyed as fast as it is formed, which accounts for it not being found in the ultimate fermentation products.

and withstands a temperature of 110° C. It is precipitated along with albuminoids by treatment with alcohol, acetone, and ammonium sulphate. It can be dialysed with difficulty or not at all, and occurs in variable quantity in the living cell according to its stage of development

The very extensive investigations of Harden and Young have contributed largely towards our actual knowledge of this enzyme, both of its composition and reactions By forcing the juice under pressure through a film of gelatine supported by the pores of a Chamberland filter it was found possible to divide the juice into a residue and a filtrate, either of which was incapable of promoting the alcoholic fermentation of glucose, whereas, when united, the mixture produced almost as active a fermentation as the original juice. The filtrate was invariably found to be quite devoid of fermentative power A solution of the residue in water is usually quite inactive to glucose or fructose. When the original filtrate or a corresponding quantity of the filtrate from boiled fresh yeast-juice is added, the mixture ferments glucose or fructose quite readily. These experiments lead to the conclusion that the fermentation of glucose and fructose by yeast-juice is dependent on the presence *not only* of the *enzyme, but also* of *another substance* which is *dialysable* and *thermostable*, in contrast with the enzyme properly so-called, which is *non-dialysable* and thermostable. That second substance, the chemical composition of which is unknown, is called the **co-enzyme.** The enzyme of zymase is then inactive. if not stimulated by the co-enzyme. Further experiment led Harden and Young to the important observation that, if **potassium** or **sodium phosphate** be added to a sugar solution undergoing fermentation by the juice, the quantity of carbon dioxide found is greatly increased, and further, that the quantity is *directly proportional* to the *phosphate added* On the basis of this observation, Harden's further work led to the interesting conclusion that the phosphorus present in the juice *takes an active part in fermentation* and undergoes a remarkable cycle of changes, which mainly result in phosphate of the co-enzyme entering into combination with the sugar, in presence of a specific enzyme, forming what Harden calls a **hexose-phosphate.** The consequence is that the phosphate is rendered inactive; but it is again liberated by an enzyme contained in the yeast-cell, **hexose-phosphatase,** which thus reproduces both sugar and phosphate So long as sugar is in excess and fermentation proceeds, hexose-phosphate will be produced, providing phosphate is present, and no accumulation of free phosphate will take place. But if the hexose-phosphate be added to the residue after filtration of the juice (*i.e.*, after the co-enzyme has been separated), then phosphate will accumulate, without any fermentation taking place. Consequently, zymase does not act directly on sugar; the phosphate of yeast-juice enters into unstable combination

with sugar, and it is only after the phosphatase has set free the sugar that zymase can function fermentatively, the liberated phosphate combining with part of the unchanged sugar and so forth *A given amount of phosphate can thus promote repeated fermentation on an extensive scale* If the juice loses its fermentative power, the cause is to be sought either in the fact that zymase has been attacked by the proteolytic enzyme, or that the co-enzyme has been decomposed by lipase In that case it is possible to re-activate the zymase by adding fresh co-enzyme or boiled juice The hexose-phosphatase appears invariably to outlast the enzyme and co-enzyme

Gay-Lussac, in 1815, produced the well-known fermentation equation, according to which one molecule of sugar is converted into two molecules of alcohol and two molecules of carbon dioxide This equation is still essentially correct At the same time small amounts of aldehyde, glycerine, and acetic acid are produced In other fermentations one molecule of sugar is transformed into two molecules of lactic acid

The problem of the **intermediates** produced in the course of fermentation, was elucidated by the thorough researches of Neuberg and several others

The work of Neubauer and Fromherz led to the supposition that **pyruvic acid** plays a prominent part in alcoholic fermentation, that this is a fermentable acid, to be regarded as an intermediate product of fermentation, leading to the formation of **aldehyde.** We are indebted to Neuberg for the important observation (1910) that pyruvic acid can be readily fermented by yeast, and also for the discovery of a special activating agent or ferment termed by him **carboxylase,*** which tends to generate **carbon dioxide**; it invariably accompanies zymase, and appears to be organically related to it By cautious heating it is possible to modify the "total zymase" in such a way that it will no longer react on sugar, but only on pyruvic acid By this discovery the important problem of the formation of carbon dioxide appears to have been solved

It was proved by Fernbach and Schoen, in 1913, that pyruvic acid really occurs in the fermentation of sugar by ordinary alcoholic yeasts As to the other fermentation products, Neuberg has propounded the hypothesis that the first intermediate product is methylglyoxal (CH_3—CO—CHO), produced in some unexplained way by a twofold abstraction of water from the sugar molecule He holds that this hypothesis is borne out by the fact that methyl-glyoxal can be produced from sugar by treatment with hot concentrated caustic soda, and also by treatment at a low temperature with alkalies, sodium carbonate or bicarbonate, ammonia, and the like.

During the reaction of alcoholic yeast upon sugar in the complete

* This ferment was found in the filtrate of a maceration juice heated to 50° C. (*cf* the co-enzyme described above).

absence of air, carboxylase causes oxygen, set free by decomposition of the water molecule, to unite with methylglyoxal. Thus pyruvic acid is produced and by the further action of carboxylase is split up into **carbon dioxide** and **acetaldehyde.** Both the formation and decomposition of pyruvic acid are assumed to go on continuously throughout the fermentation

Meanwhile, **hydrogen** from the water molecule is retained in such loose association that it is capable of reducing the acetaldehyde formed by pyruvic acid to **ethyl alcohol,** or else the hydrogen may combine with a fraction of the methylglyoxal, to form **glycerine.**

By detecting a ferment, in 1913, capable of converting methylglyoxal into **lactic acid,** the occurrence of all the principal and accessory products of alcoholic fermentation have been accounted for.

The above survey does not account for the participation of **phosphoric acid** in the fermentation process Important researches on this point were made by Harden and Young (as briefly mentioned above), and also by Euler

Neuberg found corroboration of his theory in the experimental fact, that, by addition of certain substances to the fermenting liquid, it was possible to bring about an abundant yield of some of the intermediate products referred to This proceeding is termed by him " Abfangverfahren "

As early as 1874, Dumas isolated the oxidation product acetaldehyde, by addition of sulphurous acid or its salts, such as sodium sulphite The part played by these reagents in the fixation of aldehyde, which made it possible to study certain factors of the fermentation process, was established later (1914-15), by Muller-Thurgau and Osterwalder, who used pure cultures in their very exact investigations In Neuberg's experiments aldehyde is formed in large quantity and immediately combines with the sulphite. Up to 80 per cent of the theoretical yield was found At the same time, according to Neuberg, the mobile "fermentation hydrogen," which is liberated by fixation of the aldehyde, reduces the sugar molecule to an equivalent quantity of **glycerine.** The formation of glycerine as the chief product of such a fermentation is entirely conditioned by the fixation of aldehyde This mode of fermentation, by contrast with that represented by the normal equation of Gay-Lussac, is called by Neuberg the " second type of fermentation "*

* This work has been utilised in the manufacture of **glycerine** on a large scale. A very high yield of this product was obtained by Connstein and Ludecke (*Ber D chem Ges*, 1919), by the use of large quantities of sodium sulphite, which is well tolerated by the yeast The following mixture may be used —10 litres of water, 1 kilo sugar, 400 g sulphite, and nutrient salts (ammonium sulphate, sodium phosphate, and potassium salts) After two days' fermentation 100 parts of sugar yielded 27 of alcohol, 3 of aldehyde, and 20 of glycerine, which were extracted from the filtrate after distillation of the alcohol and precipitation of the salts The yield of glycerine was increased by increasing the amount of sulphite and adding larger quantities of salts of neutral or alkaline reaction The **race** of yeast does not seem of importance, neither does temperature nor the specific sugar

If, on the other hand, fermentation is induced by another group of alkalisers, the true alkaline salts, such as sodium carbonate or potassium phosphate, the increased yield of aldehyde will be partly oxidised and partly reduced (Cannizaro's reaction), two molecules of aldehyde will yield one molecule of ethyl alcohol and one molecule of acetic acid, at the same time, the hydrogen liberated gives rise to the formation of glycerine The yield of glycerine and acid sometimes exceeds 40 per cent. of theory This mode of decomposition of sugar is termed by Neuberg the "third type of fermentation." *

Neuberg and his collaborators, by employing their "Abfang-verfahren" in a number of **bacterial** and **mould fermentations,** further demonstrated that pyruvic acid and acetaldehyde are undoubtedly formed as intermediates as in alcoholic fermentations, such is the case, for example, with *Bac coli*, *Bac lactis aerogenes*, *Aspergillus*, *Mycoderma*, *Monilia*, *Oidium*, *Mucoraceæ*, and notably with the butyric ferments In many bacterial fermentations chemical changes occur similar to those observed in the "third type of fermentation," but hydrogen is liberated

By his "Abfangverfahren" Neuberg showed that large quantities of aldehyde are produced, even in decidedly aerobic fermentations, such as the acetic fermentation

As regards the action of **acetaldehyde** on fermentation, it was shown by Harden (1921) that the process was appreciably accelerated by adding it to a mixture of lævulose, phosphate and yeast juice or zymin The same effect was obtained when methylene blue was allowed to act on a similar mixture containing dextrose and lævulose

Abderhalden (*Fermentforschung*, 5, 1922) observed that the addition of adsorbent substances, particularly animal charcoal, to dextrose, saccharose, maltose, lævulose or galactose, fermented by living cells, caused an extraordinary yield of acetaldehyde, but this was only partially due to oxidation of ethyl alcohol When fermentation took place without access of air, an extraordinary amount of glycerine was also produced, in accordance with Neuberg's theory

Thus, in chemical transformations and decompositions taking place in micro-organisms, aldehyde appears to be as essential as formaldehyde is in the assimilation of carbon dioxide in green plants

For details of the valuable researches contributed by Neuberg,

* Fermentation with sodium carbonate and molasses was carried out on a large scale by Eoff (Report, Lab Internal Revenue Bureau, Washington, 1918) with an ellipsoidal wine yeast At the height of fermentation an addition of solid sodium carbonate (up to 5 per cent) was made At a later stage ammonium chloride was also used to increase the yield of glycerine, which, however, was far inferior to that obtained in sulphite fermentation Such carbonate fermentations are obviously of practical importance only in countries where molasses are very cheap.

Fromherz, Fernbach, Schoen, Mazé, Muller-Thurgau, Osterwalder, and others towards the solution of these important problems, the reader is referred to the chemical literature.

The Enzymes of Yeast.

In addition to the general sketch included in the foregoing chapter on the enzymes occurring in fungi, a few particulars must be given regarding their special relations to yeast.

Invertase is commonly found in all species. It is prepared by treatment of the yeast with alcohol or ether, or by drying and heating to 100° C. The enzyme is then extracted with water or glycerine and precipitated with alcohol; the precipitate is afterwards dried. It hydrolyses cane-sugar, which is split into one molecule of glucose and one of lævulose, and it is only after hydrolysis that yeast can ferment sugar. It splits up raffinose into fructose and melibiose. Willstatter, however, asserts that this effect is due to a special enzyme, **raffinase.** The optimum temperature of invertase is about 55° C., it is destroyed at 75° C., but in a dry state it can withstand much higher temperatures. It is resistant to small doses of antiseptics, and to vigorous attack by the proteolytic enzyme of yeast. Its activity is increased by very dilute acid, but considerably diminished by alkali. The optimum acidity, as is generally the case, does not depend on the quantity of acid, but rather on the concentration of the dissociated portion of the acid—that is, on the hydrogen ion concentration. Sorensen's thorough researches on this subject have proved the activity of invertase to be largely dependent on that concentration, with an optimum at $p_H = 4{\cdot}4$ – $4{\cdot}6$. If living yeast is suspended in cane-sugar solution with addition of chloroform or toluene, fermentation is arrested, whilst the invertase is unaffected.*

Maltase also occurs in many yeast species. It decomposes maltose into two molecules of glucose. It has its optimum at 40° C. and is destroyed at 55° C. Whereas invertase can be extracted from the cell in aqueous solution after the yeast has been killed, it is not so with maltase. The thorough researches of Willstätter proved this to be due to the fact that certain enzyme reactions result in the formation of acid, by which maltase is destroyed as fast as it is dissolved in water. Hence maltase is confined to a narrow range of hydrogen ion concentration. If, therefore, the acid be neutralised with ammonia, it is possible, on addition of toluene or chloroform to the aqueous solution, at temperature, to extract a strong maltase solution from dried

* The influence of acids and bases on enzyme action often depends on the formation of salts in the medium. Albuminoids, peptones and amido-acids frequently have an accelerating action due to their faculty of combining with acids and bases (Sörensen's "buffer" action).

yeast (drying by exposure to air is sufficient) as well as from fresh yeast The extract also contains invertase

Melibiose is decomposed by **melibiase,** which, according to Bau, is usually present in bottom-fermentation yeast but is often absent in top-fermentation yeast

Lactase splits up lactose into one molecule of glucose and one of galactose It acts best at the neutral point (p_H about 7) Willstatter, by cultivating yeast, more particularly *Sacch fragilis* (*e.g*, in yeast decoction and lactose), found that lactase can be most readily obtained from fresh yeast by grinding for ten minutes with chloroform and subsequently diluting the liquefied yeast with water while continuously neutralising with 1 per cent ammonia. After leaving it to stand for two or three days, the lactase solution is filtered off Air-dried yeast can be similarly treated with water and neutralised, and the lactase solution separated by a centrifugal after twenty-four hours

Research by Armstrong, Slator, Harden, Euler, Willstatter, Steibelt, Sobotka, and others has demonstrated that yeasts incapable of fermenting galactose may acquire this property if grown in a nutrient liquid containing the sugar

Certain yeasts—*e.g*, *Sacch Logos* and *S Pombe*—were shown by P. Lindner to be able to ferment dextrines, bodies intermediate between starch and maltose

The combining of phosphoric acid is effected by a particular enzyme, which was examined by Euler and called by him **phosphatese;** he found that it can at any rate be partially separated from the other enzymes, and that it displays its maximum activity in weak alkaline solution As mentioned in the chapter on Theories of Fermentation, the formation of a hexose-phosphate in alcoholic fermentation was observed by Harden, who also found it to be again split up by a special enzyme, **phosphatase,** present in fairly considerable quantity in yeast juice

The **proteolytic enzyme, endotryptase,** plays an important part in the life-history of the cell, more particularly in regard to so-called auto-digestion Beijerinck, as well as Hahn and Geret, considers this enzyme to have a purely intercellular activity and to be incapable of liberation until the cells have died, Will, on the contrary, believes that his numerous experiments justify the conclusion that the liquefaction of gelatine by the action of this enzyme is a function of normal cells, under definite conditions, not yet completely known, thus, some *anomalus* cells were found to liquefy gelatine quickly in Bottcher's moist chamber, but no dead cells were found in the liquefying colonies; further, observations made by Will on yeast growth at low temperatures suggest that the proteolytic enzyme can diffuse through the cell wall. In yeast juice it was shown to exist by Buchner and Hahn. It has its optimum at 40°-45° C, and is destroyed if heated for

an hour to 60° C., it is more stable in the dry state It resists weak antiseptics and acids, neutral salts favour its action, saccharose checks its action at 5 per cent strength It is destroyed by 35 per cent strength

Buchner and Hahn, observing that boiled yeast-juice not only protects the zymase, but also the albumen of the juice against attacks by the endotryptase, believed this to be due to a special **antiprotease,** the presence of which could be demonstrated in a cold aqueous extract of acetone yeast evaporated *in vacuo.* They also showed that the co-enzyme in the boiled juice can be destroyed without detriment to the antiprotease By boiling yeast-juice for 7 to 8 hours, so as to destroy the co-enzyme, Haehn and Schifferdecker prepared a "protective juice" exerting a strongly activating influence on the zymase This juice further contains an activator of the co-enzyme, associated with an anti-lipatic agent The co-enzyme is very sensitive to **lipase.*** As weakened yeast-juice may be invigorated by admixture of common boiled juice—boiled for a short time only, and consequently still containing the co-enzyme—it may be assumed that the co-enzyme is first attacked, the zymase properly so-called still remaining undestroyed, hence it would appear that it is lipase which attacks the co-enzyme

Reducing enzymes are also found in yeast cells, and amongst these must be classed the enzyme which converts sulphur into sulphuretted hydrogen † According to Nastukoff, Osterwalder, Schander, Will, and others, it occurs in very different degrees of activity in the various yeast species

The reducing action of yeast has acquired considerable interest from recent researches on fermentation Thus, yeast juice and yeast itself were shown by Harden, Hahn and others to act upon methylene blue, which is quickly discoloured even in highly concentrated solutions Harden and Norris found that dried yeast lost its power of reducing methylene blue when it is washed with cold water and, as noted above, that it recovered this power on addition of aldehydes.

In this connection must be mentioned yeast **catalase,** discovered by O. Loew It liberates oxygen from solutions of hydrogen peroxide, and can be extracted from the cell by means of water and glycerine It occurs in yeast juice, and also in the juice obtained by autolysis of yeast

Finally should be noted the special enzyme in the cell which

* One or more imperfectly known enzymes believed to take part in the consumption and the secretion of fats in the cells

† In both beer and wine sulphuretted hydrogen may occur (in wine the disease is called "Bockser"), more particularly at the end of the fermentation As both the grapes and the wine and beer casks are treated with sulphur, it may find its way into the liquid, but even in the absence of free sulphur or sulphur compounds, the albuminoids of the nutritive liquid and the contents of the cell protoplasm may provide material for the formation of sulphuretted hydrogen Certain experiments appear to indicate that a diseased condition of culture yeasts may cause the disease

effects the conversion of glycogen—**glycogenase.** It is not secreted from the cell.

Action of the Yeast Species on Carbohydrates and other Constituents of Nutritive Liquids.

Diseases in Beer.

The first decisive proof that species of *Saccharomyces* may produce several distinct reactions on the nutritive liquid was given by means of pure cultures of yeasts prepared by E C Hansen in 1883, and by the author

Hansen's epoch-making researches on disease yeasts proved that amongst the wild yeasts there are groups which bring about detrimental changes in beer, whilst others proved to be harmless. Amongst the former there are some which impart a bitter taste and disagreeable odour to beer (*Sacch Pastorianus I*), usually without producing turbidity, whilst others (*Sacch Pastorianus III.* and *Sacch ellipsoideus II*)* only fully develop their activity at a late stage of the secondary fermentation, and then make the beer turbid. This effect is due to an abundant yeast deposit formed a comparatively short time after the finished beer has been drawn off, which rises at the slightest movement of the liquid. These disease yeasts cannot produce turbidity if they only come in contact with beer at the close of the principal fermentation It is possible, however, if the beer comes in contact with the two species after storing, that an infection with young cells of *S ellips II.* might produce turbidity The disease yeasts which influence the odour and flavour of beer are only of importance when they occur at the beginning of the principal fermentation The chief danger lies in the pitching yeast Weakly fermented beer is much more liable to attack than other beer Becker made the interesting observation that certain wild yeasts, which impart a bitter taste to beer, are capable of influencing the attenuation when mixed with culture yeasts. The fermentation is increased and in certain cases extends over a longer period than with the pure culture yeast. Wild yeasts can also bring about disturbing effects in top-fermentation breweries. For instance, according to de Bavay, the "summer-cloud" of Australian beer is caused by a *Saccharomycetes*, which causes turbidity, and imparts a bitter and slightly acid taste. In English high-fermentation beers the author found yeasts of the *Saccharomyces anomalus* type which produced turbidity, in weakly-fermented, Danish, high-fermentation beers, *Torula* species having similar properties occur. Similarly van Hest found species of *Torula* producing turbidity in top-fermentation Dutch beers Chapman found that *Sacch. Past I* occurs in

* We retain the original names of these species, giving the now accepted nomenclature in the special chapters

English beer, and gives the well-known bitter taste known as "yeast bite." Frew observed that the "stench" of English beers which have undergone secondary fermentation, due to sulphuretted hydrogen or a similar substance, is derived from a special wild yeast, *Sacch. foetidus I* It is well known in the trade that *S. ellips. II* and other species may produce disease even when the infection first occurs in the storage casks, transport casks, or bottles. Reference must be made to the fact that mixtures of culture yeasts, each capable of producing a good product, may, according to Hansen, produce disease in beer. By the use of mixtures for pitching yeast it was found that the species present in smaller quantity rendered the beer more liable to turbidity, in comparison with fermentation carried on with the leading species alone Even when the two species were separately applied, and the beers mixed for the first time in the storage casks, similar phenomena were observed

Pichi has found species producing disease in wine.

In the storage casks of lager beer, Lafar found a budding fungus of the *Mycoderma* type, which produced acetic acid.

Just as the moulds react differently upon various carbohydrates, so it has been shown by the exact researches of Hansen and others that the yeasts exhibit pronounced characteristics. In addition to the true Saccharomycetes, *Mycoderma cerevisiæ*, *Sacch. apiculatus*, the *Torulas*, and *Monilia*, are reviewed in the following paragraphs:—

Hansen's six *Saccharomycetes* (*Sacch cerevisiæ I.*, *Sacch. Pastorianus I.*, *II.*, and *III*, *Sacch ellipsoideus I.* and *II.*) behave as follows:—They all develop invertase, they convert saccharose into invert sugar and ferment the latter; they ferment maltose and dextrose, but not lactose. All the bottom yeasts used in practice react similarly with these four sugars.

Sacch. Marxianus, *Sacch. Ludwigii*, and *Sacch. exiguus* do not ferment maltose and lactose: they invert saccharose and ferment nutritive solutions of invert sugar and dextrose.

Sacch. octosporus ferments maltose, dextrose, and lævulose, but not saccharose.

Sacch. membranæfaciens and *Mycoderma cerevisiæ* possess no inverting enzyme, and do not ferment the four sugars, or only feebly ferment dextrose and lævulose

Sacch. apiculatus does not invert saccharose, and of the four sugars it only ferments dextrose. It only induces, therefore, a feeble alcoholic fermentation in beer-wort.

Amongst the *Torulas* there are many which do not secrete invertase, are incapable of fermenting maltose, and only yield about 1 per cent. by volume of alcohol in beer-wort. Other species invert saccharose. In nutritive dextrose solutions the different species induce a more or less vigorous fermentation.

Monilia candida, although possessing no inverting enzyme soluble in water, ferments saccharose, maltose, and dextrose. It ferments beer-wort, but at ordinary room temperature it only yields the higher percentages of alcohol at a much slower rate than the Saccharomycetes

In milk, various budding fungi have been found Of these, Grotenfelt, Dombrowski, and the author have described certain Saccharomycetes, Duclaux, Adametz, Kayser, and Beijerinck several non-Saccharomycetes They all decompose lactose.

Fermi found that certain red and white yeasts exercise a diastatic action Morris arrived at similar results in experiments with pressed yeast

If we now review all these different properties of the Saccharomycetes, we shall see that they fall into two groups —

I Those which possess an inverting enzyme and induce alcoholic fermentation This group is further subdivided into —

(*a*) Those which not only ferment saccharose and dextrose, but also vigorously ferment maltose the six species first described by Hansen, and the yeasts employed in the brewing industry

(*b*) Those which ferment saccharose and dextrose, but not maltose—*e g*, *Sacch Marxianus*, *Ludwigii*, and *exiguus*.

II. Those which do not possess any inverting enzyme, comprising —

(*a*) Those fermenting maltose, dextrose, and lævulose—*e.g.*, *Sacch octosporus*

(*b*) Those fermenting only dextrose, such as *Sacch. mali Duclaux*, *Sacch unisporus* and *apiculatus* varieties

(*c*) Those producing very weak, if any, fermentation in dextrose and lævulose—*e g*, *Sacch membranæfaciens*

The budding fungi which do not form endospores (non-Saccharomycetes) show the greatest variation regarding both inversion and fermentation.

I The great majority do not ferment maltose Many of these induce a more or less vigorous fermentation in solutions of dextrose and invert sugar Some *Torulas* invert saccharose, and many possess no inverting ferment (*Mycoderma cerevisiæ*, *Torulas*, and *apiculatus* varieties)

II *Torula novæ carlsbergiæ*, and a few of the species isolated by Will, ferment maltose One species (*Monilia candida*), resembling *Torula*, ferments maltose as well as saccharose and dextrose. It contains no inverting enzyme soluble in water

The lactose-fermenting *Saccharomycetes* and *Torulas* demand special classification

When we consider the behaviour of these fungi in the fermentation industries, it will be seen at once that it is only in the genus *Saccharomyces* that species occur, which rapidly and vigorously ferment maltose Yeasts for breweries and distilleries must,

therefore, be selected from the true Saccharomycetes The non-Saccharomycetes, the great majority of which cannot ferment maltose, are scarcely capable of playing any important part in these industries, on the other hand, they may be employed in the manufacture of wine from grapes, currants, and other fruit, as several species are able to induce just as vigorous a fermentation in solutions of dextrose and invert sugar as the Saccharomycetes

Amongst the carbohydrates synthetically prepared by Emil Fischer, isomaltose may be mentioned For some time it played a great part in the literature As is well known he discovered this sugar in the products of the action of hydrochloric acid on grape-sugar at a low temperature The name isomaltose signifies a constitution similar to that of maltose The sugar is known only in the form of an osazone Even the existence of Fischer's isomaltose has been questioned, as it was considered to be impure maltose By a fresh investigation, however, Fischer succeeded in proving biologically that this sugar is sharply distinguished from maltose by the fact that isomaltose is neither fermented by fresh yeast nor split up by the enzymes of yeast, and he asserts that it is only possible to differentiate with certainty between the two sugar species in this way

The different action of the *Saccharomycetes* on the same nutritive liquid (wort or must) under identical conditions, has been further studied by Borgmann, Amthor, and Marx

According to Borgmann, the chemical reactions brought about in wort by the two Carlsberg bottom yeasts, No 1 and No 2, show a striking difference These two species—which had been in use for some time in the fermenting room, and were still practically pure—were employed for pitching two fermenting vessels containing wort from the same brew; the fermentation took place under conditions which enabled a true comparison to be made, and the resulting beer was stored as usual The differences in the chemical reaction were especially noticeable in the proportion of free acid. Thus —

	No 1	No 2
Free acid (calculated as lactic acid),	0 086	0 144 per 100 c c
Glycerine, . . .	0 109	0 137 „

As a result of these experiments, Borgmann pointed out that the ratio between the alcohol and glycerine in these two beers differs from that previously found in beer, the ratio obtained from previous analyses being —

	Alcohol	Glycerine
Maximum, .	100	5 497
Minimum, .	100	4 140

whilst the Carlsberg beers gave the following ratios —

	Alcohol	Glycerine
No 1, .	100	2 63
No 2, . .	100	3 24

It must be admitted, as Borgmann remarked, that good beer may be produced by a method not open to criticism, in which the ratio of alcohol to glycerine may sink below the previously-admitted minimum.

A series of the eight different species of *Saccharomyces*, and amongst them six culture yeasts, all in absolutely pure cultures, were examined by Amthor with reference to their chemical action on beer-wort. The fermentations were conducted in Pasteur flasks of one litre capacity under identical conditions, and formed two series, one corresponding to the primary fermentation in the brewery, and the other to the secondary fermentation. The amount of alcohol, extract, the specific gravity, attenuation, glycerine, nitrogen, reducing substance, and degree of colour, were determined in the fermented worts. The tables show palpable differences in the chemical reactions brought about by the different species. The percentage of alcohol varied within the limits of 4·34 and 6·02 by volume (3·55 to 5·94 at the end of the primary fermentation), the extract from 8·27 to 11·23 (8·49 to 11·61 at the end of the primary fermentation), the attenuation from 36·7 to 53·3 (28·8 to 52·1 at the end of the primary fermentation); the percentage of glycerine showed very striking differences, and fluctuated between 0·08 and 0·15; likewise the amounts of nitrogen, reducing substance, and to some extent even the colour intensity showed considerable variations.

Hiepe drew some interesting parallels between the action of a number of culture yeasts and wild yeasts on the sugars. For this purpose he instituted fermentations in sugar solutions containing yeast decoction. He took out the first sample five minutes after the fermentation had been induced, and then fresh samples every day, till the fermentation had subsided. In each sample the amount of (1) inverted sugar, (2) fermented extract, (3) fermented dextrose, and (4) fermented lævulose was determined. In these four respects well-marked, specific differences developed in the course of a day. Thus, in five minutes an English high-fermentation yeast had inverted 1·95 per cent. sugar, whilst a low-fermentation yeast from the author's collection had inverted 58·85 per cent. A complete inversion of the sugar with two low-fermentation brewery yeasts took place in the course of about twenty-four hours, whilst in the case of *Sacch. exiguus* this reaction required eleven days; for the other species the time required lay between these two limits. The detailed tables, given by Hiepe, show that successive fermentation of the total quantity of extract, as well as that of the two sugars, takes place according to a scale peculiar to each individual species. A glance at the numerous details of the experiments further shows that the fermentation of dextrose, as a rule, begins much more vigorously than that of lævulose; but whilst the fermentation of the former

reaches its maximum on the second day, the fermentation of lævulose does not reach its highest activity until later, in some species even as late as the fifth day, by slow degrees the proportionate amounts of sugar fermented approach each other, and finally both sugars disappear simultaneously.

The yeasts also vary in the amount of acid produced in the nutrient liquid From this point of view Prior examined the fermentation products of a number of brewery yeasts and wild yeasts in hopped wort, and found that the amounts of acid formed varied from 4·7 to 10 c c. of decinormal caustic soda solution per 100 c.c. of fermented wort, the fixed organic acids varied from 2·1 to 5·4 c c, the volatile organic acids from 2·1 to 5·8 c.c. The evidence shows that, in culture yeasts, the amounts of fixed organic acids usually exceed those of volatile acids, whereas in Hansen's wild yeast species (*Sacch Pastorianus I., II.*, and *III.*, and *S. ellipsoideus I.* and *II*) the reverse is the case, the volatile acids exceeding the amount of the fixed acids; this is specially the case with *Sacch. Pastorianus I*

A large number of Saccharomycetes occurring in must were examined by Marx in 1888 both morphologically and in relation to their chemical action on the nutritive liquid. They showed distinct variation in fermentative power, and in their capacity for producing volatile substances, which impart a special bouquet to wine, and finally in their power of resistance both to various acids and to high temperature.

Amthor subsequently investigated a number of absolutely pure cultures of wine yeasts, and detected specific differences in the time taken by the fermentation, as well as in the chemical composition of the wines. Similar results have also been obtained by Jacquemin, Rommier, Martinand, and Rietsch in France; Müller-Thurgau in Switzerland, Wortmann and Nathan in Germany; Mach and Portele in Austria; Forti and Pichi in Italy; some of the comparative experiments conducted by those authors having been carried out on a large scale.

More thorough and extensive investigations into the varied action of wine yeasts on must are due to J. Wortmann. He states, as the general upshot of his investigations, that differences in the divers types of genuine wine yeast are sometimes so great that they can be detected merely through chemical analysis of the products of fermentation or metabolism, in other cases, however, they are of such a kind that we can only convince ourselves directly of their presence by their odour and flavour. Every type of yeast shows some individual peculiarity, more or less characteristic of its action on any must, regardless of its nature or origin

The number of yeast cells formed in a given must, apart from the nutritive contents of the must, depends on the specific power

of propagation of the chosen type, on the other hand, it is in itself independent of the origin of a particular must. In any given must, whether it be an excellent, or an indifferent nutrient medium for the wine yeast, one given race of yeast will multiply more freely than any other.

An extensive comparison of the amount of extract contained in a number of wines fermented with three different yeast species showed that in the same must, the "Wurzburger" yeast consumed the smallest quantity of extract, next came the "Johannisberger," whilst the "Ahrweiler" yeast used up the largest amount of extract, and accordingly left the smallest residue in the wine.

The specific activity of wine yeasts is clearly brought out in the formation of glycerine, which has a predominant influence in determining the flavour of wine. The three species mentioned above, were compared in a large number of musts of different origin, and it was found on the average, that Würzburger yeast formed more glycerine than the other two; of these, the Johannisberger yeast was superior to the Ahrweiler, which, as already stated, ferments the extract most vigorously.

The difference observed between the chemical activities of these species was emphasised by the fact that the Wurzburger yeast had multiplied most feebly. This example, *inter alia*, shows that alcoholic fermentation of must is independent of the formation of glycerine. It is, therefore, impossible to establish a definite relationship between the contents of glycerine and alcohol in wine.

Both the percentage of nitrogen and of ash proved to vary in wines fermented with the three kinds of yeast.

The acid content was highest in wines fermented with Wurzburger yeast, and was practically equal for the other two kinds.

In accurate comparative experiments, a large number of species differed widely with respect to the amount of alcohol produced in the liquid; those yeasts having the shortest fermentation period yielded the smallest percentage of alcohol, and conversely.

With regard to the bouquet in wine, Wortmann and Muller-Thurgau distinguished between that which originates in the grape—"grape bouquet"—and that which is produced as a result of the activity of yeast—"fermentation bouquet." In some wines the grape bouquet is so strongly developed that the fresh bouquet formed by the action of yeast on certain bodies present in the grape plays only a subordinate part in determining the character of the wine. In other wines, where the grape bouquet is not so strongly developed, the fermentation bouquet may greatly influence its character. The result of applying a pure culture of a wine yeast will, therefore, differ with different wines. In the first group of wines the influence must be regarded as an indirect one in that the pure culture suppresses foreign organisms, which might mask the true grape bouquet, whereas the fermentation bouquet

ll have but little influence on the wine In the fermentation of ch wines the best results will be obtained by using yeasts from e locality It is quite otherwise with the second group of wines ith such musts possessing no outstanding characteristics, the plication of specially selected yeasts will exercise a directly vourable influence on the flavour and on the whole character the wine The long experience of the author has fully established e fact that both the quantity and the variety of the fermentation uquet may be increased by such means

The fermentation bouquet differs with each yeast species, but ither its quality nor quantity stands in direct relationship to fermentative power, nevertheless a certain uniformity appears exist among races of yeast in their action on any given wine

Kayser compared the products of several types of wine yeast, d found that the formation of volatile acids at higher temperatures fered for each species. Thus, with a rise in temperature, the antity of these acids increased with one species and decreased h another

Ventre studied the action of four races of wine-yeast occurring ncipally in Southern France (Beaujolais, Médoc, Romanee, and rzenay), employed by him to set up fermentation in natural l artificial media He confirmed the results arrived at by earlier rkers—namely, that the fermentation products of one and the ne yeast vary according to the composition of the particular pe must in which it is grown He then studied the question the fermentative power possessed by each particular yeast, and this purpose he used :—

(1) Must, both in the fresh state and sterilised,

(2) A neutral nutrient liquid containing 170 g of glucose, 2 extract of malt-culms, 0·5 g malt-peptone, 0·5 g yeast ash, di-ammonium phosphate per litre,

(3) An acid nutrient liquid containing like the foregoing liquid, g glucose and so forth, but having an acidity of 5·3 g per e, expressed as sulphuric acid, but produced by adding 4 g. taric acid, 5 g malic acid 0·5 citric acid, with 1·85 potassium bonate

Each of the four species was characterised by a marked difference the yield of glycerine and succinic acid, and also in point of enuation The Médoc yeast yielded a higher amount of dry ract in the wine than did any of the other races, yet the extract not contain any residual reducing substance Some of species showed a marked propensity to produce volatile ls The fixed acids in the liquid were attacked differently each species.

Concerning the use of pure cultures in practice, Ventre draws following conclusions :—A thorough knowledge of the chemical vities of the different species is of essential importance in

making a selection. As an example of the value of organoleptic characters, he points out that in hot countries, where lack of acidity is the general rule, it is advisable to use yeasts like that of Champagne, which are capable of preserving the freshness of the product, whereas in cold climates, where the acidity is generally high, it is reasonable to select yeasts that tend to lower the acidity.

The numerous investigations carried out continuously, since 1884, in the author's laboratory, with pure cultures of yeasts, as applied in the various branches of the fermentation industry, have furnished ample opportunity for collating experience relating to the chemical activity of species, and their respective powers of *retaining their peculiarities intact during storage*, a matter of importance in every branch of the industry. Numerous instances have been met with in which even feebly-pronounced characters, manifested through taste or smell, remain inherent after several years' preservation of the culture under proper conditions, they can even be restored by skilful development of the culture under favourable conditions

The Products of Alcoholic Fermentation.

It has already been stated that saccharose can only be fermented after the intervention of invertase has caused its hydrolysis into glucose and lævulose. The same holds good with regard to maltose, which is split up into two molecules of glucose In a similar way lactose is hydrolysed by certain species of yeast before alcoholic fermentation takes place * Other sugars (the hexoses) are directly fermentable Of these, the commonest is glucose or dextrose (grape-sugar), which is fermented by every known species of alcoholic yeast This also applies to lævulose or fructose, which is so widely distributed in the vegetable kingdom, and usually occurs in conjunction with glucose

The principal product of fermentation is alcohol, more particularly ethyl alcohol In 1815 Gay-Lussac first established the true character of the reaction when he showed that cane-sugar (more correctly grape-sugar) gave 51·11 per cent of alcohol and 48·89 per cent of carbon dioxide on fermentation Pasteur showed that by-products always occur, and that part of the sugar is utilised for the nutrition of the yeast, so that it is never possible to convert the whole amount of sugar into alcohol and carbon dioxide Pasteur's results were 48·3 per cent of alcohol and 46·4 per cent. of carbon dioxide, which agrees well with recent determinations, showing that virtually equal quantities of alcohol and carbon dioxide are formed

* According to researches by Willstätter, certain yeasts are capable of *directly* fermenting maltose and lactose, respectively In fact, by determining the length of time required for different enzymatic reactions, he found that fermentation set up by yeasts poor in maltase or lactase greatly outruns hydrolysis

Rayman and Kruis proved that beer which had been subjected to fermentation with absolutely pure cultures, and kept for some years at normal temperature, contained only ethyl alcohol, but when air was introduced and the yeast formed a film, the alcohol was decomposed into carbon dioxide and water

Glycerine occurs in varying quantities, and, according to Wortmann and Laborde, this does not depend entirely on the decomposition of the nutritive liquid, but more particularly upon the yeast species Its production is favoured by a high temperature of fermentation, and by a greater sugar concentration, or in general by the use of a rich nutritive fluid. It is almost impossible to give limits for the proportion of alcohol and glycerine. In wine fermentation the yield of glycerine varies usually from 2·5 to 14 per cent. of the amount of alcohol produced, whereas in beer it represents only 1·65 to 4·3 per cent of the alcohol By fermenting saccharose with zymase Buchner and Rapp obtained even smaller quantities of glycerine On the production of larger quantities of glycerine under special circumstances, see the chapter on Theories of Fermentation

It was shown by Pasteur that succinic acid is formed in varying quantities as a by-product of alcoholic fermentation. As indicated by F Ehrlich, this acid is not a direct result of the decomposition of sugar, but proceeds from the metabolism of the albuminoids of yeast, and originates from the glutaminic acid formed in the fermentation process The amount of the acid produced depends largely on the state of the yeast

Lactic acid is found as a by-product in fermentations carried out with yeast-juice or with living cells

Prior's detailed researches proved that the different races of yeasts produce varying quantities of volatile and non-volatile acids. He found that acetic acid was a constant product of fermentation An abundant production of volatile acids, more particularly acetic acid, was observed by Osterwalder in Sicilian must fermented by certain wine-yeasts

Formic acid is produced, according to Rayman and Kruis, by the oxidising action of yeast on the albuminoids of the nutritive liquid.

Aldehyde (acetaldehyde) also occurs regularly Rayman and Kruis proved that, especially in the case of distillery yeasts, considerable quantities are formed when free access of air is permitted, and the surface of the fermented liquid is then covered with a film of yeast. Muller-Thurgau, in the whole course of wine fermentation, also found aldehyde as a product of decomposition of the sugar. Under normal conditions it is immediately converted into alcohol, but it can be isolated in the course of fermentation by combination with potassium metasulphite.

Methyl alcohol, often found in bacterial fermentations, may

also be developed during a yeast fermentation, especially by the fermentation of glucosides present in fruit juices. It also appears possible that propyl and butyl alcohol may be produced in a normal alcoholic fermentation, the former from lactic acid Especial interest is attached to the presence of amyl alcohol (isoamyl alcohol), which forms the main constituent of fusel oil; according to Rayman and Kruis, it is produced in larger quantities at high temperatures and in the absence of air. Amyl alcohol is freely produced in liquor containing grains which have been treated with sulphuric acid According to Ehrlich, fusel oil is formed in the ordinary growth of yeast from leucin and isoleucin, two cleavage products of albumen. It appears to be produced also in the auto-digestion of yeasts.

We must also record the production of acetic ether and other volatile and non-volatile ethers which help to impart a specific character to the fermented liquids

Auto-fermentation.

Pasteur's researches indicated that yeast is capable of forming alcohol and carbon dioxide under certain conditions, even in the absence of sugar from the surrounding liquid. By boiling yeast with dilute sulphuric acid, he prepared a fermentable sugar which he believed to be derived from the cell-wall

Salkowski has proved that in reality glycogen plays a part in auto-fermentation. Salkowski states that, by treatment with chloroform-water, glycogen is split up, but auto-fermentation does not take place. In the opinion of Harden and Paine, the velocity of auto-fermentation is increased by any substance exerting a plasmolytic action on the yeast cell. Thus acceleration is effected by a solution of 5 to 6 per cent of common salt or ammonium sulphate, whereas, in deci-normal solutions, these salts have no influence The normal fermentation of glucose, on the contrary, is greatly retarded by addition of common salt Beijerinck, experimenting on a dough made of 2 parts of pressed yeast and 1 part of water, found the maximum development takes place at 48°-49° C, at 30° it was very slow. Auto-fermentation will go on until the whole of the carbohydrate has been consumed; the cell will then die.

It is not only the carbohydrates, but also the nitrogen compounds, that are gradually resolved in the yeast cell. A continuous demolition and re-formation of albuminoids takes place in the cell. Under unfavourable conditions, such as lack of nitrogenous nutriment, or oxygen, high temperature, or the presence of toxic substances, demolition will prevail or even entirely suppress construction, and thus there will take place an *endo-proteolysis* and *autolysis*—self-digestion—whereby albuminoid

substances are decomposed by endotryptase and peptase This often occurs while auto-fermentation is going on. A number of decomposition products are thus produced—guanine, adenine leucine, tyrosine, ammonia, etc—as has been shown by the addition of toluene or chloform to protect the yeast against attack by bacteria. The process results in a complete destruction of plasma. A rapid autolysis can be obtained by placing pressed yeast in closed Petri dishes at 48°-50° C, the yeast will be liquefied in a few hours

In practice, auto-digestion may take place in the manufacture of pressed yeast, where decomposition of the yeast is frequently encountered without the occurrence of any bacterial infection. In this case, owing to lack of nourishment, the cells gradually degrade their albuminoids The yeast mass is then more readily exposed to infection by bacteria Cells rich in glycogen appear to be less liable to such decomposition.

Fermenting Power ; Fermentative Energy ; Raising Power.

The work carried out by yeast can be distinguished under three heads.—Activity of the enzymes, metabolism, synthesis of material

The activity of the enzymes is of a sugar-splitting, or hydrolysing, and proteolytic character As proposed by Neumann Wender, the fermenting power of yeast may be expressed in terms of the quantity of sugar which is split up at a given temperature in unit time by unit quantity of yeast The fermentative energy may be defined by determining the time required to decompose a given quantity of sugar by unit mass of yeast under specific conditions The "raising power" is a function of the carbon dioxide evolved by pressed yeast, whereby the dough is raised

The following are examples of determinations of this kind :—

Fermenting Power.—Evolution of CO_2 in twenty-four hours from 1 gramme of yeast and 40 c c of 10 per cent. cane-sugar solution, at a temperature of 30° C

Fermentative Energy.—Evolution of CO_2 in the 1st, 2nd, 3rd, and 4th half-hour, from 10 grammes of pressed yeast in 400 c.c. of 10 per cent cane-sugar solution at 30° C

In the case of flour or meal, the strength is estimated by the following process :—

Three grammes of yeast are dissolved in 60 c.c. of distilled water at a temperature of 30° C, and a dough kneaded by intimately mixing up with 120 g of wheat-flour The dough is placed in a graduated cylinder of 1 litre capacity and 8·5 cm. clear width, and exposed for 4 hours at 30° C in a water-bath, to promote fermentation. The initial volume of the dough and the time of placing it in the cylinder must be exactly noted, and for two hours, the volume produced should be read off every half-hour. With

the aid of a smooth wooden pestle, the dough is then reduced to its initial volume, and again allowed to ferment for two hours, the expanding volume being read off every thirty minutes

Baking Tests are at present carried out with 280 g of flour, 160 c c of a salt solution containing 4 g chemically pure sodium chloride, together with 2 g. of sugar and 5 g yeast

The sugar is dissolved in the saline solution at 30° C, and the yeast then stirred in The resulting mixture is thoroughly kneaded with flour for ten minutes, quickly rounded by hand, and put into the slightly greased baking tin at a temperature of about 30° C (the dough must not be squeezed into the tin) The tin is immediately placed in the fermenting case, the temperature of which should be kept at 33°-35° C

The dough is first observed an hour after kneading is begun. The time taken for the rising dough to reach a rule placed across the tin is noted in due course.

Exactly two hours from the beginning of kneading, the dough is baked 30 minutes at 230°-250° C in a laboratory oven

The Biological Relationships of Yeast.

The problem of the occurrence of yeast in nature was raised as soon as its vegetable character had been established The first researches on this question were undertaken by Brefeld in 1875, who concluded that the yeasts are very widely distributed in nature, and that their germs are present in atmospheric air, in dust, and in vegetable matter, and that their breeding places are specially to be sought in the excrement of herbivorous animals It will be seen from what follows that this view can no longer be accepted It is true, as the author has proved by his own investigations, that the excrement of herbivorous birds contains numerous budding fungi, and amongst them *Saccharomycetes*, but their breeding places must be sought in quite a different direction

In 1876 and 1879 Pasteur published complete memoirs regarding the occurrence of yeasts on grapes, and stated that they were to be found only on ripe grapes At the same time he did not succeed in answering the important question as to where the yeast fungi found a habitat during the remaining part of the year. He expressed the view that *Dematium pullulans*, which is found everywhere on grapes, lives through the winter in the form of thick-walled and coloured resting-cells, and produces new yeast cells in the following summer, but it is now recognised that these budding cells are not wine-yeast cells On the other hand, it was shown by the author in 1895 that other mould forms occurring on grapes, which resemble *Dematium*, but do not possess thick-walled resting-cells, produce internal spores which develop budding *Saccharomyces* cells What part these moulds play in the preservation of the yeast vegetation has not yet been determined.

Great uncertainty still existed regarding the all important ·oblem of the habitat of the yeast during each season of the year. , was solved for a single species by E C. Hansen in 1880-81, ıd further research, of a detailed and fundamental character, eared up the question for so many other species that this ıportant phase of the biology of yeasts is now fully understood. he researches of Hansen were first carried out on the small ,mon-shaped yeast-fungus, *S apiculatus*, which always appears the earliest stage of wine fermentation. By microscopical ɛamination and culture experiments it was shown that during ıe summer months the organism appeared in vast quantities ıth the ripening of the sweet juicy fruits (cherries, gooseberries, ,rawberries, grapes, plums, etc). On the other hand, it was ıly quite exceptionally that it was found on the unripe fruit. s the organism was found vigorously budding on the ripe fruit ›ecified, but never, or only very rarely on other fruit, and on eir leaves and branches, the fact may be accepted that these pe fruits act as true hosts to *S apiculatus* This was further tablished by the observation that it is to be found without ɛception in the soil under cherry and plum trees, vines, and ;her fruit-bearing trees upon which the organism grows, but ıat it is extremely seldom found in samples of soil taken in ,her localities of a most varied character. The fruit falls to the ·ound, and the rain washes the fungus into the soil, the problem, ıen, is whether it is able to hibernate there The answer was ɔtained in two ways First, numerous samples of soil were taken ıring the course of the winter and spring at these localities, ıd in the vast majority of cases gave a vigorous growth of e organism in wort. Secondly, cultures of *S apiculatus* were aced with every precaution in the earth, and allowed to remain roughout the winter. They were removed in the spring and ,rly summer, culture experiments proved that the organism ıs alive in every sample In this way it was established that e organism is able to hibernate in the earth, just as it had been ·eviously shown that it only occurred in the soil of these particular ːalities In later experiments of Hansen's, vigorous cultures the organism were placed in the surface soil in well-sealed ıamberland filter tubes. Three years later the contents of these bes were introduced into sterilised wort, and a vigorous growth the organism developed The life-cycle of the fungus may, erefore, be spread over more than one year.

It still remained to be proved whether the earth is the true ıbitat in winter This was determined as follows —Hansen ·amined dust in a great variety of places from January to June, so the dried fallen fruit of many trees, and lastly, many nds of excrement These analyses gave a negative result, and ıus furnished the desired proof The soil under the specified

fruit trees must, therefore, be regarded as the true winter habitat of the fungus. It preserves its usual appearance throughout the long winter, and is then carried into the air by the combined agency of insects and wind, and by such means of transport is distributed from fruit to fruit.

It is obvious that during the period when a large number occur on ripe fruit air currents may carry the fungus to other localities, and also on to unripe fruit Hansen stated in his first memoir that the rare occurrence on unripe fruit must be due to the fact that the organism quickly dies off, partly through want of nourishment, and partly through the drying up of the cells He subsequently proved by experiment the correctness of this view. He distributed both old and young cells in water, and spread them either in a thin layer on an object glass or on a tuft of thinly spread cotton-wool; thus allowing evaporation to go on while the cells were protected from the sun In less than twenty-four hours the whole of the cells were killed It is quite obvious that individual cells spread over the surface of unripe fruit are exposed to more unfavourable conditions than in his experiments If, however, thicker layers of cells are covered by cotton-wool or filter paper, they remain living, just as they do in the soil, for a long time. Thus they survive for more than eight months in filter paper.

It was then possible for Hansen to demonstrate that the great majority of yeast species must pass through a similar cycle in nature. Their most important breeding places are the sweet juicy fruits. Their winter habitat is the soil, and they are carried by wind, rain, insects, and other creatures on to the fruit. They then multiply once more on sweet fruit, and obviously more particularly where juice oozes out from the fruit. Hansen further found that these yeast species often occur in the ground at places far removed from orchards, where *S. apiculatus* can no longer be detected.

Muller-Thurgau arrived at the same results as Hansen with regard to *S apiculatus* during an examination of wine species. He found that grapes are their chief breeding places, and that their presence may be detected in soil throughout the year. On the other hand, they seldom occur in the air. He further proved that cells of wine-yeasts may occur in soil at a depth of from 20 to 30 cm.

In 1897, Wortmann's researches, recorded in his work on the preparation of wine, were directed to determining the life-history of wine yeasts in soil at different seasons of the year The experiments were continued for two years, and consisted in taking samples of soil every fourteen days from one and the same part of a vineyard. By sowing the soil in sterile must, he obtained an idea of the flora. His main observation was, that directly after the vintage (in November and also in December), the samples of soil in must developed a growth of yeast so rapidly that no other

fungi were able to develop. In January, February, and March also a development of yeast was always obtained from the samples, but it occurred more slowly. In spring and summer the conditions were always less favourable, and a longer period elapsed before fermentation began. Some samples, indeed, gave no yeast development, but only other organisms. The least favourable conditions were observed in the late summer (August and September), but from the time the grapes began to ripen, vigorous growth was again observed in the flask. Wortmann concluded that while the wine yeast survives in soil, its nutritive state is of the greatest importance. Vegetation is most vigorous during the early stages, when it has been enriched with cells fresh from grapes—*i.e.*, in autumn, winter, and the beginning of spring—whereas during summer, the most favourable period for vegetation generally, its activity is constantly diminishing, the cells having drawn upon their reserve material. According to this view, yeast is dependent upon its own body-material during its habitat in the soil. The lower temperature ensuing after the vintage allows metabolism to go on so slowly that it enables cells to survive throughout winter and spring.

In early summer, with increasing temperature, the cells rapidly assimilate the remainder of the reserve substance, and consequently die off slowly. Cells that are still alive are weakened, and samples of soil, therefore, give a very feeble growth in the flask. Cells are continuously carried by insects and other means from the soil to the vegetation, and those which light upon the grapes when they are ripe find full nourishment and produce a new vigorous growth. Wortmann was able to confirm Muller-Thurgau's observations, that no wine yeasts are to be found in a vineyard which has not been worked for a long time; they are gradually exterminated by exhaustion. In those wine districts where the culture of grapes has been continued for centuries, the yeast cells which are conveyed from soil when the grapes are ripe adapt themselves more and more to the excellent nutrient material, and in this way specially good races of wine yeasts are developed.

In 1903 and 1905 Hansen obtained results which differed from those of Wortmann in an important point relating to the condition of yeast cells during their abode in soil which the latter regarded as a state of starvation. This new and very detailed research led to the result that elliptical and *Pastorianus* forms of *Saccharomyces* (but not *S. apiculatus*) are to be found throughout the year in all kinds of soil in the neighbourhood of Copenhagen. Their number diminishes, however, at a distance from the orchards. A similar condition of things was found by examining soil in the Harz Mountains and in the Alps. The soil in vineyards is specially rich in yeast species, and the greater the elevation the smaller is

the number of organisms found Above a certain height no yeasts are found

The reason for this wide distribution lies, as Hansen showed, in the fact that, in addition to the normal breeding places for yeast, there are others which he called secondary breeding places —*e g* , aqueous extracts from fruit and other vegetable matter, and from excrement In the former, the cells multiply very rapidly, in the latter, feebly or not at all. If yeast cells from sweet juicy fruit and from the upper layers of soil, where they form spores, are carried by insects or by wind to distant places, they may, unlike *S. apiculatus*, maintain life even when dried, on account of their greater power of resistance In the same way they can multiply more readily in soil in the aqueous extracts already referred to, and may even preserve life for a longer period in presence of nothing but moisture Thus the fact is fully explained that the larger species occur much more widely distributed throughout the soil than the small lemon-shaped wine yeasts *S anomalus* and *S. membranæfaciens* are especially resistant to the effect of drying. They are, therefore, found at great distances from the primary habitats In this way the fact may also be explained that fewer yeast species are sometimes found in the soil of vineyards than in the neighbouring meadows The cells in the vineyards are dried up and killed, whereas in the meadows where the cells are protected from drying, life is maintained, and the cells multiply In such places cells also occur during the hot season of the year, and here their propagation goes on most vigorously Where the ground is subject to drought the variation brought about in the course of years may be altogether extraordinary

The soil must, therefore, be considered the chief habitat of yeasts at every time of the year They are carried from the earth by means of wind and rain, as well as by the action of insects and other creatures, to the sweet juicy fruits, where they multiply vigorously , a few fall to the earth again, whilst others are carried to secondary places of incubation

When the fruit is ripe the wild yeasts thus strongly developed find their way into the fermentation industry. It is only if they are allowed to remain, to multiply, and to obtain a secure footing, that they are capable of bringing about any disturbance in the industry Otherwise they are immediately suppressed by the large quantity of the culture yeast added to the nutritive liquid

During their development on grapes and other juicy fruit the yeast cells compete for nutrition with many other organisms, including bacteria and moulds These observations led Wortmann to adopt the view that the true importance of alcoholic fermentation is biological Most of the competitors of yeast can multiply much more rapidly, and would soon suppress it if no means existed for restricting their growth. This means is supplied by the alcohol

produced by the yeast cells, whereby they are able to poison their enemies Wortmann showed how the poisonous action of alcohol is apt to support yeast in competition with other organisms. During the early stages of the development of yeast in must a surface growth of various organisms can be observed Among these the small apiculate yeast is especially prominent, and this soon brings about a fermentation The alcohol so formed suppresses most of the moulds The true wine yeasts now gradually begin to develop, and simultaneously the development of *Mycoderma*, of bacteria, and of the *Dematium* species, ceases As soon as the alcohol content rises above 4 per cent, as a result of the activity of the true yeasts, *S apiculatus* is suppressed, and the wine yeasts immediately take command of the field to such an extent that, in an ordinary microscopical examination, nothing but their cells can be observed The most powerful alcohol-formers amongst the yeasts again gradually supersede the weaker species

Temperature plays a great part in the life of yeast cells, and Hansen has made use of this relationship as one of the most important means for characterising the species

In 1883 he proved that both the spores and vegetative cells of different species possess different powers of resistance to heating in water In this respect the spores are more resistant than the vegetative cells

In such determinations the condition of the cells has a marked influence, and the result depends largely upon their age. Thus the two-day-old cells of *S ellipsoideus II*. grown in wort at 27° C. were killed on warming to 56° C. for five minutes in sterilised distilled water, whilst cells similarly prepared, but two and a half months old, were heated to 60° C for five minutes without being destroyed

Ripe spores of this species, developed at 17°-18° C, and partially dried for eight days at the same temperature, withstood heating for five minutes at 62° C, but not at 66° C

The vegetative cells of *S. cerevisiæ I*. were killed by five minutes' heating at 54° C, and the spores at 62° C.

An interesting classification of Hansen's six species in relation to any given temperature is obtained by cultivating them in wort under conditions favouring the formation of films. Thus, if the development is carried out at 36°-38° C, the three *Pastorianus* species are killed in eleven days, whilst *S. cerevisiæ I* and the two ellipsoid species remain alive From this and similar experiments, it may be argued that the rule formerly accepted that top-fermentation yeasts can develop at a higher temperature than bottom-fermentation yeasts has no general application

Kayser's more recent work along the same lines has confirmed these results. He also proved that the species withstand considerably greater heat in a dry than in a moist condition. Thus

a yeast species isolated from pale ale was killed in a moist condition by heating for five minutes at 60°-65° C., whilst in a dry condition it withstood a temperature of 95°-105° C., and in the case of a wine yeast (St Emilion), the corresponding temperatures were 55°-60° C and 105°-11° C. The spores withstand temperatures 10° and even 20° higher than the vegetative cells.

Vegetative cells which are derived from the heated spores show a somewhat greater power of resistance than normal vegetative cells This increased power of resistance is not transmissible, by cultivation in beer-wort it disappears entirely in the second generation

The temperature limits within which budding of cells can take place in wort were investigated by Hansen The upper limit for *S. Past I* is 34° C, for *S. membranæfaciens* 35°-36° C, for *S. anomalus* and *S Ludwigii* 37°-38° C, for *S Past. II*, *III*, and *S. ell. I.*, *II*, and for *S cerev. I* about 40° C, and for *S. marxianus* 46°-47° C The lower limit for each of these species is 0 5° C, with the exception of *S. cerev. I* and *S. Ludwigii* with a limit of 1°-3° C. Muller-Thurgau found that the wine yeasts that he examined are incapable of propagation at temperatures above 40° C

It is, of course, impossible to establish any one temperature that shall serve as the optimum for the growth of yeast cells, because the composition of the nutritive liquid is of more importance than it is in other relations The formation of new cells in the same liquid goes on at a diminishing rate when the development proceeds at a constant temperature, because the increasing quantity of the products of metabolism and the simultaneous impoverishment of the nutritive fluid acts restrictively upon the growth, especially at higher temperatures An approximate temperature of 28°-30° C is found to be favourable for the development of many species. Without doubt the species behave differently in this respect as well as in regard to the maximum production of yeast which can be developed from a given inoculation

Many fermentations take place in the industry at lower temperatures, indeed, in the case of bottom-fermentation breweries, very considerably lower than the optimum for the multiplication of the cells. In order that fermentation may be completed at so low a temperature within a reasonable time, and before other organisms have an opportunity of infecting the liquid, relatively large amounts of yeast are introduced, and propagation is assisted by aeration At times the pitching yeast is first placed in a smaller quantity of the liquor at a higher temperature (about 20° C.), allowed to grow for a few hours, and the newly-formed and vigorous cells are then introduced into the cold liquor There appears to be a tendency to forego the extremely cold fermentations once customary in many places In distilleries, where fermentation

proceeds at a higher temperature, it is often necessary to take special precautions to avoid a considerable rise in temperature during the first stages of the fermentation, otherwise the propagation of cells ceases too soon Consequently the growth would be so enfeebled that it would be impossible to carry the fermentation to completion

Variations in the Saccharomycetes.

The numerous investigations by Hansen and other *savants* proved that the Saccharomycetes are affected in varying degree by external agents, and that it is possible by suitable treatment to bring about variations along different lines Even the individual peculiarities of cells in a pure culture may be of importance in this respect Some of these changes are only evanescent. By suitable cultivation they disappear, and the species returns to its original condition Others are more deeply seated, and it is only by a special treatment that the culture can be deprived of its newly-acquired properties In certain cases, it is found impossible, even after years of methodical treatment, to cause a growth to revert to its original state

1 The times given for the appearance of the first indication of spores are based upon the understanding that the growth has been cultivated at 25° C for twenty-four hours in wort In 1883 when Hansen published temperature curves for his six species, he found that growths which had been developed for two days instead of one, at the same temperature, developed spores more slowly and less freely then usual If, however, they are subsequently treated in wort in the way described, the normal conditions are re-established This forms an example of a very feebly-rooted variation

2 In a gelatine culture, Carlsberg bottom yeast No 1 is often found in both oval and elongated sausage-shaped cells. If a colony derived from each of the cell forms is transferred to flasks containing wort, a growth is again obtained consisting partly of oval and partly of elongated cells Hansen's experiments proved that the latter when cultivated in new flasks retained to some extent the sausage-like form, and when transferred to the pure culture apparatus the growth continued to show a mixture of such cells, but when the yeast was then conveyed to an ordinary fermenting tun they disappeared The variation in this case is, therefore, a more deeply seated one It only ceases when the yeast has been transferred through a series of fermentations Another example is shown by a bottom yeast which, after a long period of stunted growth, had been propagated in wort at about 27° C, and formed cells with a normal appearance, whilst the growth cultivated at 7·5° C gave entangled colonies with mycelial branchings. This

forms a striking example of the effect that temperature has upon the form of cells

3 Hansen's observations of *S Ludwigii* supply an illustration of a far-reaching change in the character of the cells. If single individuals are grown as pure cultures, growths are obtained which show a marked difference in their power of spore-formation By systematic selection of single cells, Hansen succeeded in producing growths which gave no spores under the usual conditions, and conversely, it was possible to select a yeast colony derived from a cell containing spores, and by further cultivating the colony to obtain a growth which possessed the power of freely generating spores. By such systematic choice the species was divided into three forms—one distinguished by its vigorous spore-formation, another by the fact that this power had almost disappeared, and a third, which could not form spores. By frequent infections in wort the third form reverted to the power of forming spores This took place slowly, but when Hansen transferred it to a 10 per cent dextrose solution with yeast decoction this property was instantly restored.

In other species, varieties which have lost their power of spore-formation completely, or in part, may make their appearance, without any known cause, both in liquid and on solid nutrient media. In some cases (*e.g.*, *S. Ludwigii*) that power is restored if dextrose is added to the nutrient liquid Similar observations regarding asporogenesis have been recently made by Beijerinck on *S. octosporus*

If a pure culture of brewery yeast is developed in a wort which has not been aërated after sterilisation, it generally loses its normal "breaking" and clarifying properties, under brewery conditions, and this to a degree dependent on the species. These new variations must often be cultivated through a great many generations in ordinary brewery wort before regaining the original qualities of the species. As aëration brings about changes in the chemical composition of the wort, it is evident that the effect on the protoplasm is due to such circumstances

The author of this book showed in 1890 that when a brewery top-fermentation yeast which has given a good clarification in practice is kept for some time in wort-gelatine at room temperatures, it tends to lose its clarifying properties for a considerable time. At the same time, it brings about a considerably stronger attenuation than in its original condition

As an additional instance of the effect of the chemical composition of wort in producing new varieties, we may mention the observation, due to Hansen, that *S Pastorianus I*, which imparts an unpleasant taste and smell to beer-wort, is apt to lose this power for a time if preserved in an aqueous solution of cane-sugar

A similar proof of a variation in brewers' low-fermentation

yeast, due to the composition of the nutrient liquid, was furnished by Seyffert, who found in the case of a selected type which, after long use in breweries, had lost its good properties with regard to clarification, that it was possible to restore it to its original condition by treatment with lime Gypsum was added either to the wort, the brewery water, or the steeping vat, and from wort prepared in this way wort-gelatine was concocted, in which the degenerated yeast growth was sown for fresh pure-cultivation. On development of the colonies in small flasks, these new growths showed true "breaking" and the power of adhering to the bottom of the flask, the qualities thus regained were retained during the use of this yeast in practice

Another example of physiological transformation is the following —The three species described by Hansen under the name *Saccharomyces Pastorianus* form a dough-like sediment under certain conditions similar to those of the other *Saccharomycetes;* under other conditions, however, a film-like, wrinkled, or caseous sediment consisting of small lumps (Pasteur's *levûre caséuse*)—a sediment of very different appearance In the latter case, the fermenting wort also assumes a characteristic appearance, and, contrary to what ordinarily occurs, remains bright throughout the fermentation, so that yeast flakes may be observed rising to the surface and sinking again to the bottom. If this curious sedimentary yeast is repeatedly cultivated by new fermentations in wort, it can be again transformed into the dough-like condition.

Both Hansen and the author established the fact that by long storage under ice, and subsequent growth in wort, a brewery bottom-fermentation yeast exhibited top-fermentation phenomena, which, however, by continued pitching gradually but entirely disappeared Similar observations have been made by Will.

We also find a transitory physiological transformation in film-formations of the *Saccharomycetes.*

4. In 1889, Hansen published the results of a series of experiments which were undertaken with the hope of discovering the conditions causing variation, and of experimentally bringing about the formation of new races, and if possible new species He has since published additional work on the subject.

(*a*) He found in the case of typical *Saccharomyces* that when their cells were cultivated in aerated wort * at a temperature above the maximum for their spore-formation, and near the maximum for their vegetative growth, they were affected in such a manner that they lost their power of forming spores and films (*Asporogenesis*). This was also true of the innumerable generations successively formed in new cultures under the most varied conditions. The starting point was always a growth which showed not the slightest trace of asporogenous cells. For example, it may

* By repeated shaking of the successive cultivations.

be noted that *S Past I.* loses its power of forming spores by ment at 32° C. In the case of the wine yeast, *Johannisbe* this occurs at 36° C In the seventh culture of *S. Past I.* cells were asporogenous. Hansen succeeded also in bringing a transformation by cultivation on solid media. Such asporog growths were formed in the case of *S Past I* on wort-gelat 32° C, when inoculations were made at shorter intervals, the case when liquids were used

In some of the species treated in this way, it was also obs that they yielded a more abundant crop of yeast in wort-cul but a slower fermentation. This was, for instance, the case Carlsberg low-fermentation yeast No 2 The newly-formed v attenuated more slowly and weakly than the original species at the same time the clarification was better

Rayman and Kruis have shown that the cells present in possess the power of oxidising alcohol produced during ferm tion, into carbon dioxide and water Hansen's varieties, completely losing the power of forming films, are rendered inca of performing this oxidising action. Thus, while a flask, cont the original species which had developed a luxuriant film six months' standing, showed only 1 5 per cent by volume of al a parallel flask, which showed no film-formation, contained 5 cent of alcohol—a quantity equal to that found at the end first month

In another series of experiments Hansen showed that the of higher temperatures upon the cells without aeration was ca of producing radical and lasting alterations of a different k the nature of the protoplasm. When Carlsberg yeast No. cultivated in wort at 32° C. through eight cultures, each succ culture being inoculated from the preceding one, which had left undisturbed until the end of the fermentation, a variety evolved in the ninth culture which produced 1 to 2 per cen volume less alcohol than the original form, in wort of 14° B containing 10 per cent. of saccharose The new variety cla better under brewery conditions, and gave a weaker attenu at the end of the primary fermentation, a similar beha was noted in the case of other species

(*b*) Hansen also succeeded, by cultivation in nutrient gel in producing new stable varieties.

Thus, two varieties of Carlsberg low-fermentation yeast N each generation of which was transferred to the surface of gelatine, attained a fermentative power superior to that o original forms. The difference is still more marked when cul are developed from spores of the top-fermentation yeast *S cere I.* on yeast-water gelatine. The new varieties produced cent. more alcohol than the parent form.

The observations already detailed regarding asporogenesis

ie interesting conclusion that a species can lose one of its icteristic properties as a result of external influence, and that ally a new species is produced

1 the course of Hansen's experiments on spore transformations ght about by the action of temperature and aeration, it was ved that if cells of successive generations were removed, many affected even in the first growths under the new conditions; modification, however, is temporary in character, it is only successive generations have been allowed to develop through nued inoculation under the new conditions, that the acquired icters become constant It appears from this that the transation does not depend on temperature or aeration alone, but on the nutrition and propagation of the cells

omparison of these factors has however shown that they ibute unequally to the result Both nutrient liquid and ion are only of importance in bringing about vigorous, fresh ative growth, and may, therefore, vary within wide limits out materially affecting the result This, however, is not true mperature, a fluctuation of a few degrees is sufficient to ent the variations described Hence, it follows, that temperaplays the principal part in bringing about such transformations.

s previously stated, these remarkable changes are only brought t by long-continued and violent interference with the vital sses of the cells, they do not occur so long as development place in the normal manner.

n example of the way in which the *Saccharomyces* cells retain power of forming spores under ordinary conditions is supplied eweries and distilleries Here culture yeasts have existed nuously for centuries, and untold generations have been prod under conditions which would not allow, as a rule, of this ion being brought into play, and yet the power remains t

ansen observed a remarkable variation when young growths *ellips II.* and the wine yeast *Johannisberg II.* were preserved few months in Freudenreich flasks in shallow layers of wort 5° C A few cells of these bottom-fermentation yeasts gave ermentation phenomena Further investigation showed that tion had taken place The top-fermentation yeast cells ined continuously as top yeasts, the bottom-fermentation cells as bottom yeasts A similar state of things was observed ig the examination of a large number of cells from old cultures rewery bottom-fermentation yeasts On the other hand, ar cultures both of wild top-fermentation and of brewery ermentation yeasts yielded only a small number of cells which ayed bottom-fermentation phenomena. We are not dealing, fore, as in the previous cases (asporogenesis) with the action finite factors producing a transformation, but with unknown

causes, and probably with sudden variations of the same kind as the mutations studied by H de Vries. According to these researches the two physiological forms, top- and bottom-fermentation yeasts, are not independent On the contrary, they may both occur in a growth derived from an individual cell They can exist together in the same liquid, one or other securing the upper hand in their competition and thus determining the character of the growth.

Since 1887 the author has pursued as one of his principal problems the study of the variation of yeasts during their application to the different branches of the fermentation industry His investigations must now be numbered by the thousand The difficulty in work of this character, where large masses of yeast are under observation, is to make sure that the cultures grown from a given number of isolated cells, with abnormal characteristics, are real varieties of the parent cell, and have not actually been derived by infection with foreign organisms

Botanical and biological investigation can never form more than part of a complete analysis, and must, moreover, be carried out with the utmost care To a great extent we must take refuge in the different characteristics that are developed, partly during large-scale fermentations and partly during parallel, small-scale fermentations in the laboratory For such experiments it is obvious that only yeast masses can be used which have been derived from a single cell

As a result of these observations it has been definitely established that variations do frequently take place They occur without obvious cause, and on occasion may occur in such quantities that the whole mass of yeast changes its character or "degenerates" This expression, which is used in practice, only indicates that the yeast mass, in the special brewery or distillery concerned, no longer suffices for the particular requirements It does not indicate what the true value of the yeast may be for this branch of the industry Thus occasionally such a yeast mass, that has "degenerated" produced excellent results when applied at other places where the requirements are different Selection of a cell from the yeast mass that has degenerated has often proved a true basis for regeneration, in that the new culture possesses all the properties of the original stock

A very cautious treatment of a sample of purely cultivated yeast will throw some light upon this question If a number of cells are separated from a yeast mass derived from a single cell, which has been in use in the industry for some time, the pure cultures from these cells will show differences in a set of parallel fermentations, and sometimes important differences in respect to taste, smell, and other characteristics of the fermented liquid; also as regards the attenuation, the character of the yeast layer, etc Varieties may, for instance, occur which produce a penetrating

and unpleasant bitter flavour, but in every other respect giv result in agreement with the culture yeast In this connectio is interesting to record a case where a selected variety g considerably more rapid clearing than the original race, whils every other respect, practical and biological, it was identical studying a number of selected growths a series of intermediate fo could be detected, and by a proper selection cultures were prepa which gave the normal attenuation in wort of the same charac

A problem of great practical and theoretical importance is decide whether such variations occurring in the yeast mass practice are stable or of a purely transitory nature Hansen adop the view that, as a rule, "the races prepared from industrial ye cannot be maintained, but disappear," and that "so long as beer yeasts are kept under brewery conditions, they only disp slight alterations, which are of a transitory character." This vi however, is in contradiction to the results repeatedly obtained the author's laboratory. Strongly marked abnormalities may oc when single cells are isolated, and certain of these variations pr to be of a stable character both when applied on the la scale, and also when stored for years in a 10 per cent cane-su solution There are variations still kept in the laboratory wh after preservation in such a solution for more than ten years s retain their properties These races, therefore, do not disappear.

It follows that in the preparation of pure yeast cultures be used in a brewery, distillery, wine fermentation, etc., cannot reckon on dealing simply with a type ready to ha in a pure condition, but rather with a mixture of elements, oft of a highly different character, even if the mass of yeast has be originally derived from a single cell. By the process of pure cul vation based upon a detailed knowledge of the special practi requirements, a form can be prepared of the required type. Su work can never be attempted at random, but must consist systematic research carried out with rigid rules How long su a type may be preserved in practice before it develops pronounc varieties in such quantities that the character of the yeast m experiences a change, depends to a great extent upon circumstan which are still unknown.

It will be seen from this that the principle applied in t author's laboratory in carrying out the pure culture of brewer distillery, and wine yeasts, etc , is based on a reliable starting poi and the experience gained during the long time that has elaps since the laboratory was instituted has only served to confirm t correctness of the author's view.

The *improvement* of yeast, on which the author has publish his views, consists in selecting cells taken from a mass of yea which has given satisfactory results, and preparing growths whic display the desired characters in greatest perfection. This trea

ment is carried on through several generations, and in each case after the mass of yeast has been applied for some time in practice

These observations have no connection with any variation in the composition of a nutritive fluid. They are simply concerned with comparative experiments with selected and absolutely pure cultures.

Morphology and Anatomy of Yeast Cells.

Yeast Deposits.—Hansen's investigations in 1881-1883, which took the form of a direct study of the growth of a single cell under the microscope, and of growths derived from a single cell, made it possible for the first time to give exact descriptions of the different species of yeast He proved that the shape, relative size, and appearance of the cell are not sufficient in themselves to characterise a given species, for the same species may exist in different forms under differing external influences At the same time he established the fact that the shape may provide valuable indications, as the various species may react in a different way and with a different shape when the same influence is brought to bear

As an example of the results which may be obtained by a comparison of young deposits of yeast, the six varieties isolated by Hansen may be quoted (*S. cerevisiæ I*, *S Pastorianus I*, *II*, *III.*, *S ellipsoideus I.*, *II*)

The growths are developed in the following manner —The cells, after short cultivation in wort, are introduced into fresh wort, and brought to vigorous development at 25° to 27° C in twenty-four hours If then *S. cerevisiæ I* is compared with the three *S Pastorianus* species, the general appearance is strikingly different. *S. cerevisiæ I* consists predominantly of large round or oval cells, and *S. Pastorianus* chiefly of elongated sausage-shaped cells, but it is a very different matter if the cells of the first are mixed with cells of one of the second species It then proves to be impossible, by simply noting the form, to distinguish between the larger and smaller oval and roundish cells of *Pastorianus* and many of the *cerevisiæ* cells The two species, *S ellipsoideus I* and *II.*, are predominantly oval and round. Sausage-shaped cells occasionally occur, and here again it is impossible, simply by studying the form, to determine the species when *S. cerevisiæ* or *S. Pastorianus* are mixed with them.

By direct measurement of the sedimentary forms it is also impossible to discriminate between them.

On examining the diagrams of these six pure cultures, it will be seen that we are dealing with three different divisions of budding fungi, one of which is represented by *S. cerevisiæ I.*, the second by the three *Pastorianus* species, and the third by both the *ellipsoideus* species. So much and no more can be established by a purely

microscopical observation, and this only under the particular culture conditions described

The development of the yeast cell takes place through **budding,** a slight swelling appearing in the mother cell, which increases in size As soon as the new cell has attained a certain size it can form a new bud, and this process of budding continues until a group of budding cells is formed The cells may break away from each other at an earlier or later stage, so that the group may consist of a varying number of individuals The daughter cell may assume a totally different form from the mother cell. This may also take place in the industrial species, including those which give fairly uniform oval cells in the large fermenting vats. For example, ordinary brewery, low-fermentation yeast may, for reasons unknown, produce cells with the appearance of *Pastorianus* and *ellipsoideus*, so that it is impossible, under the microscope, to distinguish whether such a culture yeast is infected with a foreign yeast or not

As an example of the change of form brought about by a known cause in industrial yeast, it may be mentioned that, by excessive treatment with air, the air-yeast of the pressed-yeast factory may alter from an oval or elliptical to a much elongated *Pastorianus* shape

In general, it may be stated that low-fermentation yeasts form groups containing fewer cells than is the case with top-fermentation yeasts. There are, however, many exceptions to this rule. It is impossible to indicate any universal type of microscopical picture for the two groups of yeasts, and the same holds good for the general picture of a single race of culture yeast. It is only by exactly comparable growths carried out in parallel experiments in the laboratory that it is possible to establish differences between the general appearance of the races When applied in practice, so many different factors come into play that the appearance of the growth may entirely alter its character On these lines no starting point can be found for an analytical examination of yeast to determine its purity

A peculiar group of yeasts, the *Schizo-saccharomycetes*, are distinguished from others by the formation of daughter cells through division of the mother cell, a cross-section being formed in the latter

Film Formation.—It is well known that fermenting and fermented liquids are covered with film growths It was first shown with certainty by Hansen's observations on cultures derived from single cells that *Saccharomyces* (in the strict sense) are able to form films.

The universally occurring *Mycoderma* species form films easily and rapidly Some also give fermentation phenomena, others do not. Such a film is greyish on beer and wort, with a dry appearance, and in its later stages wrinkled and lighter in colour. Amongst

the cells there is a considerable admixture with air Similar films are formed by a few of the *Torula* cells The film of *Chalara Mycoderma* is gelatinous, and has a bright appearance In the case of *Monilia*, which may occur with budding cells, the film formation is peculiar During the vigorous fermentation, a film forms on the froth, which gradually spreads over the whole surface, and is occasionally wrinkled The cells in the flask form a deposit, produce a vigorous fermentation, and rise with bubbles of carbon dioxide to the surface again, where they begin a new stage of development If sterilised lager beer is inoculated with this fungus, no fermentation takes place, and a thin dusty film is formed, but under other circumstances the fungus forms white, floury, and woolly layers like *Oidium*

The films of true *Saccharomyces* differ somewhat from these As a rule, they are produced in the following way —If cultures are allowed to stand undisturbed for a longer or shorter period in wort at room temperature, it will be found that small specks of yeast appear on the surface of the liquid at the completion of the primary fermentation These collect together at a later stage to form islands of varying size and shape, with a flat upper and arched lower surface Finally these fuse together to form a light greyish-yellow and slimy film, which often spreads up the wall of the vessel forming a complete ring Such a complete film-formation only takes place when the primary fermentation is completed If the flask is shaken, shreds of the skin are loosened and sink, and in this way a complete layer may be collected on the bottom, whilst the skin reforms and assumes a mottled appearance, the younger portions being thin and dark, whilst the older are thick and pale in colour

The necessary condition to enable the film to form is the presence of a free and undisturbed surface with access of air A vigorous film-formation assumes a free access of air The function of film-formation is subject to the same conditions as the formation of endospores

Along with film-formation a bleaching of the wort takes place, which now assumes a light yellow colour This occurs more rapidly at a high temperature, and is most readily observed in those species which bring about the most vigorous film-formation Erlenmeyer flasks half-filled with wort and covered with filter paper are admirably adapted for such cultures A few drops of a young and vigorous growth of yeast should be introduced.

Hansen undertook the following determinations :—

(1) The temperature limit for the formation of films

(2) The approximate time required for the first appearance of the film at different temperatures.

(3) The microscopical appearance of the growth at different temperatures.

The main object of comparative observations of this kind lies in determining the microscopical appearance of films at similar temperatures

The examination of the film was undertaken when it had just developed sufficiently to be visible to the naked eye

A glance at the illustrations representing these film-growths (see description of species) will show that their general character differs from that of the sedimentary forms. For instance, the sedimentary form of *S cerevisiæ I* is oval or spherical, whilst in the film, elongated and mycelial cells quickly appear, and the growth gradually assumes an appearance quite distinct from that of sedimentary yeast

If we compare the film-formation of the six species, we find that the films developed at the higher temperatures offer very little scope for discrimination, *S cerevisiæ I.* and *S ellipsoideus II.* alone being distinguishable from the remainder It is quite otherwise, however, when young films developed at 13°-15° C. are examined The two species, *S Pastorianus II* and *S. Pastorianus III*—both top-fermentation yeasts, the cells of which in ordinary cultures cannot be distinguished from each other with certainty—exhibit in this case entirely different forms of growth. An equally striking difference is found between the otherwise similar species, *S ellipsoideus I.* and *II.*

Observations of the limits of temperature for the formation of films show that for *S cerevisiæ I* and *S ellipsoideus I.* these lie approximately within 38° and 5°-6° C., the limits for the three *Pastorianus* species are 34° and 3° C.; *S. ellipsoideus II.* has the same lower limit as the last species, but its maximum temperature is 38°-40° C

The time limits, compared with those given for ascospore-formation, show that in both cases development takes place more slowly at low than at high temperatures. At temperatures near maximum and minimum, the film-formation is very feeble and incomplete

At temperatures above 13° C. the film of *S. ellipsoideus II.* develops so rapidly and vigorously that flasks containing this yeast can be recognised by this alone. Thus, at 22°-23° C the film had completely covered the surface in six to twelve days, whilst the other five species required three times as long to form a film, and this was generally more feebly developed This species and *S Pastorianus III* also develop a vigorous film with comparative rapidity at ordinary room temperature, the other species being left far behind.

A further important biological relationship is shown by Hansen's investigations which proved that the temperature maximum for budding in wort is higher than the maximum for film-formation, and that this again is higher than the maximum for spore-

formation, in other words, with a rising temperature, a point is reached at which spore-formation ceases, then a higher point at which film-formation ceases, and lastly, a still higher at which budding is no longer possible On the other hand, the experiments indicate that the temperature minimum for film-formation is lower than that of spore-formation

In brewers' low-fermentation yeasts, and in some wild yeasts, Will observed round and oval cells, having a thick membrane and containing a number of small oil-drops These occurred in the rings of yeast and in the small surface patches preceding true film-formation If treated with concentrated hydrochloric acid,

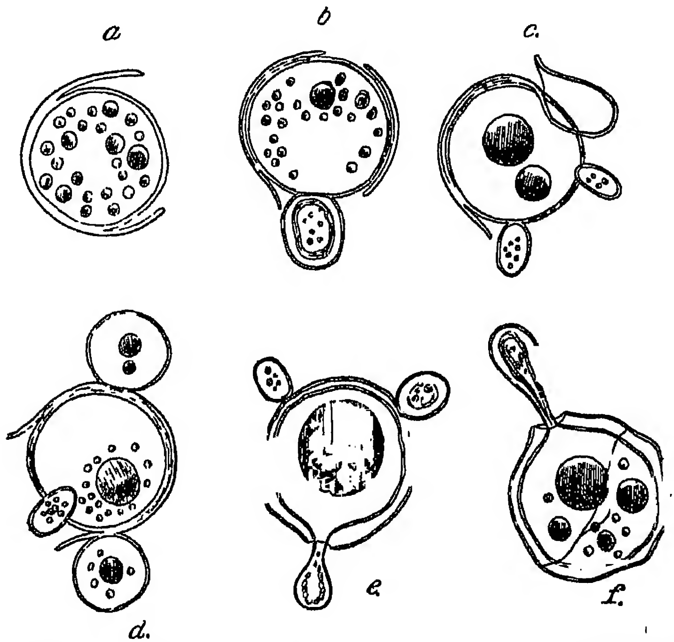


Fig. 48.—Resting cells (after Will)—The outer layer is partly or completely detached. *a*, *b*, in wort, *c-f*, in mineral nutrient solution

the membrane splits into two layers. In cultures, especially in artificial nutrient liquids, the outer layer of this membrane gradually detaches itself, sometimes in such a way that it is not torn, so that it appears as though the one cell were contained within the other. The cell contents are coloured green or brown by concentrated sulphuric acid. The glycogen reaction with iodine has been occasionally observed in the cells. They appear to play a certain part in the life economy of the growth, as resting cells, for they are sometimes found alive in old growths when most of the other individuals have perished. In artificial nutrient solutions containing mineral salts, sugar and asparagine, with

addition of citric or tartaric acid, such resting cells occur also in the sediment Globular or oblong yeast-cells germinate from the resting cells, either singly or in large numbers Club-shaped cells with transverse-wall formation frequently arise, especially in older cultures of resting cells produced in mineral nutrient solution.

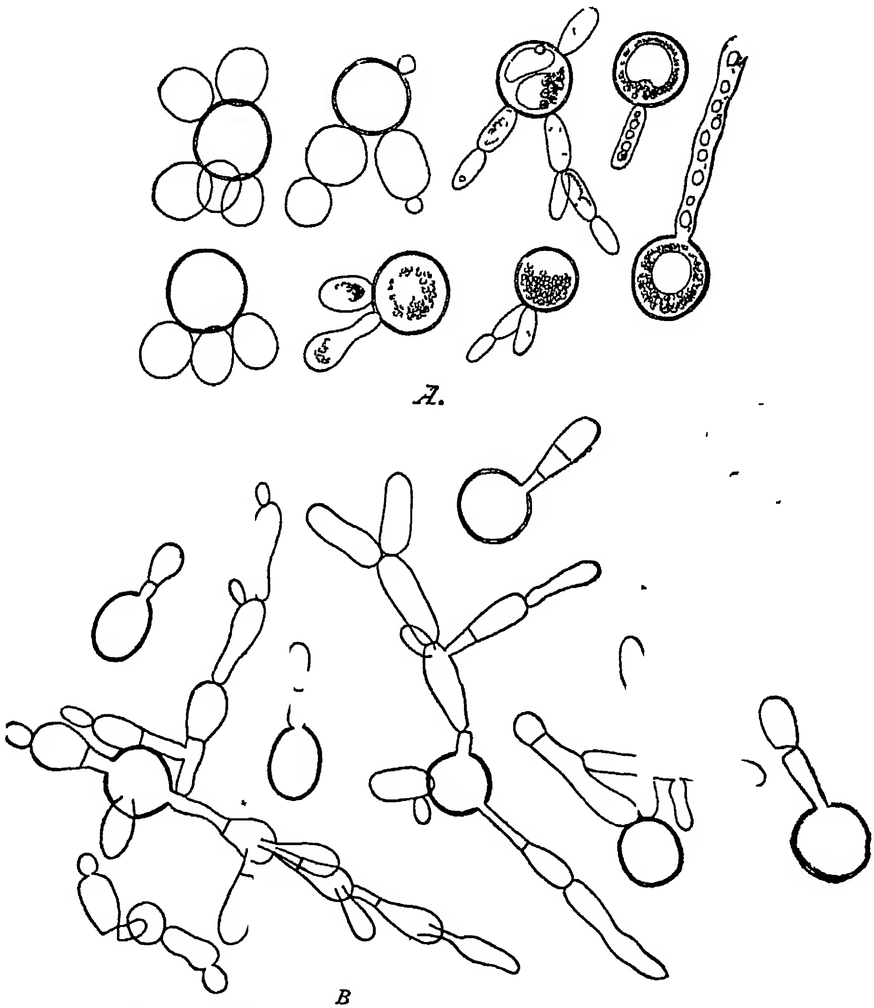


Fig 49 —Resting cells (after Will) —*A*, usual mode of germination, *B*, resting cells, with club or sausage-shaped daughter cells with transverse walls

This phenomenon may recur in derived growths During germination on a solid nutrient medium, Will also observed a splitting up of these transverse walls

According to Rayman and Kruis the cells of the film have a

marked respiratory power, oxidising the alcohol formed into carbon dioxide and water, and at the same time splitting up the albuminoids of the liquid into amides and ammonium salts of organic acids.

Cultures on Solid Substrata.—After Schroeter and Koch had shown, by cultivation on solid media, that species of bacteria display distinct characteristics, Hansen succeeded in proving that a similar relationship holds good for yeasts For this purpose he utilised beer-wort, to which about 5·5 per cent of gelatine had been added, contained in flasks closed by means of cotton-wool plugs. When these flasks were inoculated with the six species (*S. cerevisiæ I*, *S. Pastorianus I., II*, *III*, *S ellipsoideus I*, *II*), and allowed to stand at a temperature of 25° C, the growths which developed (streak-cultures) showed such macroscopic differences in the course of eleven to fourteen days that four groups might be more or less sharply distinguished *S ellipsoideus I* stands alone, for its growth exhibits a characteristic net-like structure on the surface, which enables it to be distinguished from the other five by the unaided eye When gelatine with yeast-water is employed for such cultures and the experiments conducted at 15° C, *S Pastorianus II.* yields growths after the lapse of sixteen days, the edges of which are comparatively smooth, whilst the growths obtained from *S Pastorianus III* are distinctly hairy A microscopical examination shows that the two species are also distinguishable morphologically. This is by no means always the case with cultures on solid media, in fact, the differences are often less marked under such conditions than when nutritive liquids are employed.

For the *Mycoderma* species and *S membranæfaciens*, Hansen discovered a characteristic behaviour on wort-gelatine in which they form shield-like colonies readily distinguishable from those of the *Saccharomycetes*

In this connection we may mention Hansen's observation that some species—*e g*, *S. Marxianus* and *S Ludwigii*—can develop a mycelium when grown on a solid medium, while others are unable to do so

The characters which can be obtained in this way fluctuate greatly, for the constitution of the living material and of the substratum may strongly modify the appearance of the growth This fact was brought out by Will in a special study of four species of bottom-fermentation beer-yeasts He discovered that when the inoculating material contained cells from the film they exercised considerable influence on the appearance of the colony

The giant colonies investigated by P Lindner and Will have been described in Chapter I

Aderhold, during an examination of gelatine-growths of German ellipsoid wine yeasts, found that in puncture-cultures and giant-cultures two types were distinguishable, one of which showed colonies with funnel-shaped depressions and with marked concentric

lines, whilst the other showed conical growths with indistinct concentric structure, but very prominent radial streaks

A great number of yeast species liquefy nutrient gelatine. This was proved by the author in 1890 with respect to brewers' high-fermentation yeasts Subsequently Will, Wehmer, and others made the same observations with other yeasts

Structure and Character of Yeast Cells.—During the growth of the cell the membrane gradually becomes more distinct When the cell is fully grown the strength of the membrane depends on the concentration of the nutritive fluid It has a tendency to thicken in liquids with a high percentage of extract, especially marked thickening is met with in the resting cells occurring in films The cellulose of the membrane (hemicellulose) was found by Salkowski not to be of uniform constitution If heated for some length of time with water at 2 atmosphere pressure it splits up into a rubber-like mass, insoluble in water and giving no iodine reaction, and a soluble part, precipitated by alcohol and giving an intense brownish-red reaction with iodine It is this "erythro-cellulose" which is converted into dextrose in the autolysis of yeast

The gelatinous network first observed by Hansen may be regarded as a special development of the membrane, reminiscent of the zoogloea formation of bacteria Under certain conditions, which have not yet been defined, colonies brought about by the budding of yeast cells may combine to form irregular clots which sink more rapidly than individual cells ("break" and clarification in the brewery) This doubtless stands in relationship to a feature of the development of the yeast cell discovered by Hansen in 1884 He found that both Saccharomycetes and other budding fungi may secrete a gelatinous network which may take the form of strands or plates in which the cells are embedded. If, for example, some thick brewery yeast is placed in a glass and allowed to remain under cover in such a way that it slowly dries, and then a trace of this yeast is mixed in a drop of water, the network can be clearly seen The formation also occurs in the gypsum block and gelatine cultures The author has frequently observed this formation in the yeast samples despatched to his laboratory in filter paper enclosed in envelopes * Hansen also found it in the film-formations of nearly all species An ordinary microscopic examination of the pitching yeast in a brewery does not show this formation, with the help of staining, however, its presence can be readily detected When the yeast is repeatedly washed, it is no longer possible to detect the network by staining; but if the water is removed, and the yeast set aside for a time

* This method of preserving a sample of yeast is very convenient A small piece of filter paper is rapidly passed through a flame several times, and a few drops of yeast are poured on to it, it is then folded up, and afterwards wrapped in several layers of paper which have been similarly treated

and then suitably treated, the gelatinous masses can be readily seen By varying the conditions of nourishment of the cells, the development can be promoted or retarded, and the chemical composition modified

The most important part of the cell contents is the **nucleus** which has been the subject of much research Various staining methods are described in the first chapter According to recent work by Fuhrmann, Guilliermond, Henneberg, Hoffmeister, Janssens, Kohl, Wager, and others, the nucleus in yeast fungi is of a simpler structure than in higher plants, but divergent views are held

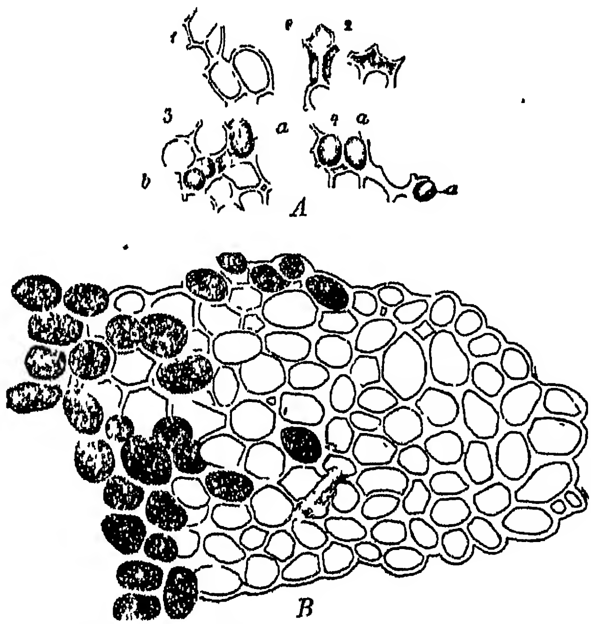


Fig. 50.—Yeast cell with gelatinous network (after Hansen) —*A*, Network obtained by partial drying; 1, portion formed of threads from which the cells have become detached; 2 and 3 show that the network can also form complete walls, such a formation is seen between *a* and *b*—*a* is a vegetative cell, *b* is a cell with two spores, 4 shows three cells, *a*, embedded in the network *B*, network with yeast cells, the latter stained by methyl violet, network is not stained Some of the yeast cells are still in the meshes, but most have detached themselves

regarding the details of the structure In the first stages of fermentation the nucleus in a normal cell consists of a **vacuole,** which is coloured less intensely, and a deeply stainable body, attached laterally to the vacuole, this body, **nucleolus,** in the course of cell life, is seen to assume different shapes—round, ovate, sickle-shaped, etc The vacuole varies greatly in size, according to the state and activity of the cell, and when the cell is least active, it apparently disappears Strong magnifications show fine granulated cords (*chromatine*), extending from the nucleolus through

the vacuole to the border of the latter According to some authorities the whole organ is provided with a membrane As in the case of all other plants, the nucleus is doubtless the centre of all the more important chemical changes and vital functions of the cell. Henneberg observed movements of the nucleus by coloration with a very dilute solution of methylene-blue or by treatment with dilute acetic acid (0 5 per cent), after keeping fresh yeast under water one or two days at about 30° C

In the budding of the cell, the nucleus generally divides—by simple division, amitose—commonly only after the bud has attained its full development, the nucleolus, growing longer, takes the shape of a dumb-bell, whereupon the two parts separate, the thread-like connecting piece being re-absorbed, one of the two parts then migrates into the bud

By a fusion of the two cells the two nuclei also merge into each other, and on large vacuole will form in the cells

Before spore-formation the nucleus divides successively into as many nuclei as spores will be formed. In a few yeasts Guilliermond observed a development somewhat similar to nuclear division in higher plants, the so-called mitose or caryokinesis, the nucleus forming a system of threads assuming a spindle shape; in the middle—that is, the widest part of the spindle—a plate is formed, the two daughter cells will form by splitting-up of the middle region.

An essential part of the contents of the nucleus consists of **nucleoproteids,** which are of the utmost importance to the life of the cell. the presence of which can be proved microchemically by submitting the cell to the action of pepsin (dissolved in 0·2 per cent hydrochloric acid), which attacks the other albuminoids of the cell

Vacuoles constitute another essential part of the cell. They separate gradually from the protoplasm of the young cells, and appear in increasing numbers as pale, feebly refractive specks. In the older cells they are sharply defined, and may assume highly irregular forms In the *Mycoderma* species one or two very large vacuoles are usually found, and this applies also to the old *Saccharomyces* cells The vacuoles are filled with an aqueous liquid

The yeast cells also contain larger and smaller particles of different refractivity, both in the protoplasm and in the vacuoles, which are classed together under the name of **granules.** They are produced even in quite young cells At a later stage, when the cells are filled with glycogen, they are not so obvious, but they are sharply defined when the glycogen has disappeared

These bodies may consist of albuminoids, fats, or carbohydrates. A group of them have acquired particular interest from the fact that, like the corresponding bodies in bacteria, they are coloured in a peculiar manner by **methylene-blue,** contrasting with other

granules. They were the subject of extensive research by Guilliermond (who discovered them in 1902), Henneberg, Kromer, van Herwerden, Zikes, and others They were called **meta-chromatic**, or **volutine bodies** by A Meyer, who found them in *spirillum volutans* and certain other bacteria They occur, in very varied size, both in the plasma and the vacuoles In the course of the multiplication of the cell, they occur as small grains and drops, increasing in size in the resting cells, they disappear gradually when the cell is kept a long time Their size and position are fixed by treating the cell with formalin, after removal of the latter, the cell is coloured with methylene-blue (1 per cent dissolved in 25 per cent alcohol), after removing the excess 1 per cent sulphuric acid is added. The granules are thus stained blue, violet, or reddish-violet, whilst all other parts of the cell are unstained By coloration of the living cell—*e g*, by the method employed by Zikes (30 c c of saturated alcoholic methylene-blue solution + 100 c c. aqueous solution of 0·1 per cent potassium hydrate), the granules enclosed in the vacuoles, in some of the species, are coloured blue, and in others red. Most authorities consider that these granules

Fig 51 —*S Ludwigii* —Nucleus division during spore-formation (after Guilliermond).

are to be classed as reserve material of the cell; their main constituent is an albuminoid, probably one of the nucleo-proteids. Phosphoric acid and nuclein have been detected Experiments have proved **phosphates** to be essential for their formation, for on cultivation in media destitute of phosphates they disappear from the cell The amount of glycogen was found by Zikes to increase more quickly in the course of the development of the cell than the volutine, which, in turn, decreased slowly

The **oily** or **fatty** bodies occurring in the cell are generally conspicuous by their strong refraction. They vary greatly in size, and increase with the age of the cell Some yeasts are distinguished by the cell containing a big central oil-drop While some of these bodies appear to consist exclusively of oils or fats, others are of a more complex composition, as they also contain albuminoids. On treatment with 1 per cent. osmic acid these bodies are stained brown or black

Glycogen also constitutes an important constituent of the cell contents It has already been stated that it is stored up by the cell when it has a rich supply of available carbohydrates. Its

presence can be distinguished by a reddish-brown coloration with iodine in potassium iodide, whereas the albuminoid substances of the cell assume a yellowish colour On heating the cell, the brown colour disappears, but reappears on cooling It seems to be stored up in special vacuoles In the fifth section of this chapter it has been shown that glycogen plays an important part in the auto-fermentation of yeast

Ascospore Formation.—In 1839 Schwann discovered that yeast cells can form new cells in their interior, and that these are liberated by the bursting of the wall of the mother-cell De Seynes gave a clear description of spores in 1868, and in 1870 Reess proved that they are produced by yeast cells of different shape, and that the germination of spores takes place by budding In 1872 Engel indicated moist gypsum blocks as a specially favourable substratum for the development of spores Reess, who did not work with pure cultures, regarded these spore forming yeasts as a special group, which he indicated by the name *Saccharomyces*, a name proposed by Meyen, but he included along with these a large number of species in which no endogenous spore-formation had been observed Similar conclusions were published by de Bary in his celebrated work *Vergleichende Morphologie und Biologie der Pilze* (1884), which also contains admirable observations regarding yeast fungi

In 1882-3, Hansen undertook the first experimental investigations concerning spore-formation, and his work made it possible to establish a sharp limit to the group of *Saccharomyces*. The results of his investigations concerning the necessary conditions for spore-formation may be shortly stated as follows —

1. The cells must be placed on a moist surface and have a plentiful supply of air

2. Young and vigorous cells can exercise this function most easily and rapidly. Old cells which lack nutritive material can only develop spores with free access of oxygen

3. The optimum temperature for most of the species yet examined is about 25° C This temperature favours spore-formation in all known species

4. A few Saccharomycetes likewise form spores when they are present in fermenting nutrient fluids.

A lack of food cannot, as Klebs assumes, be regarded as a direct condition for spore-formation, since young and well-nourished cells can also be induced to form spores immediately—without previous budding—when they are placed under conditions which favour spore-formation, but are unfavourable to budding—*e.g*, in water saturated with gypsum, but with access of air and at a favourable temperature.

A growth of yeast is developed in the way described under "yeast deposits." Older cultures, developed in saccharine solu-

tion or in wort, must be cultivated several times in aerated wort before showing a normal formation of spores A small quantity is transferred to a previously sterilised gypsum block, this block takes the shape of a truncated cone, it is enclosed in a flat glass dish covered by a larger inverted dish, and is kept moist by half-filling the dish with water * If it is desired merely to bring about the formation of spores, the apparatus may be allowed to remain at the ordinary room temperature The transferred cells develop through a few generations by means of budding, and then spore-formation begins in the mother-cells

Hansen was the first to give an accurate description of the structure of spores and a detailed account of their evolution founded upon observations of individuals. He distinguished three typically different groups of Saccharomycetes which are characterised either by their mode of germination or by the form of their spores (*S anomalus*, etc)

After a lapse of time, dependent on the species, roundish particles of protoplasm appear in the cells, these are the first indications of spores. In their further development they are surrounded by a wall, which is more or less clearly defined in the different species

In most species the spores are spherical *S anomalus* forms an exception with its hemispherical spores, *S Marxianus* and *S. fragilis* with kidney-shaped spores

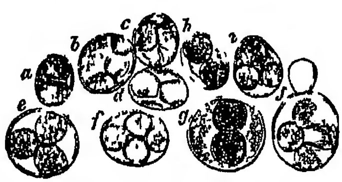


Fig 52 —The first stages of development of the spores of *Saccharomyces cerevisiæ I* (after Hansen) —*a*, *b*, *c*, *d*, *e*, rudiments of spores, where the walls are not yet distinct, *f*, *g*, *h*, *i*, *j*, completely-developed spores with distinct walls

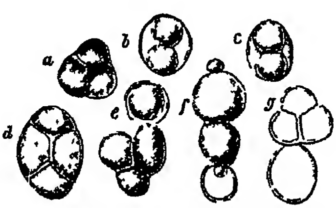


Fig 53 —Spores of *Saccharomyces cerevisiæ I* in the first stages of germination (after Hansen) —At *a*, *d*, *e*, and *g*, formation of partition walls, *e*, *f*, and *g*, the walls of the mother-cells have become ruptured, *g*, a compound spore divided into several chambers, the coherent wall is ruptured in three places.

Two distinct types of germination may be distinguished In the first type, to which *S cerevisiæ I* belongs, the spores may expand during the early stages of germination to such an extent that the pressure which they exert on each other, while they are

* *Ascospores* can also be obtained when yeast is spread upon sterilised solidified gelatine, prepared with or without nutritive solution (or on filter paper), and kept in a damp place, likewise in yeast-water and in sterilised water Spore-forming cells may also occur in the films of the Saccharomyces Klocker found certain species formed spores only when grown in dextrose-yeast water He states that it is advisable, when a species is examined with respect to spore-formation, to let the development take place both at 15° and 25° C.

still enclosed in the mother-cell, brings about the formation of partition walls (Fig 53) This is caused by the wedging or squeezing together of the protoplasm between the spores, otherwise the walls of the spores may be brought into close contact During further development, a complete union of the walls may take place, so that a true partition wall results, the cell then becomes a compound spore divided into several chambers

During germination (Fig 54) the spores swell and the wall of the mother-cell, which was originally fairly thick and elastic, stretches out and consequently grows thinner It is finally ruptured, and then remains as a loose or shrivelled skin, partially covering the spores, or it may gradually be absorbed during germination

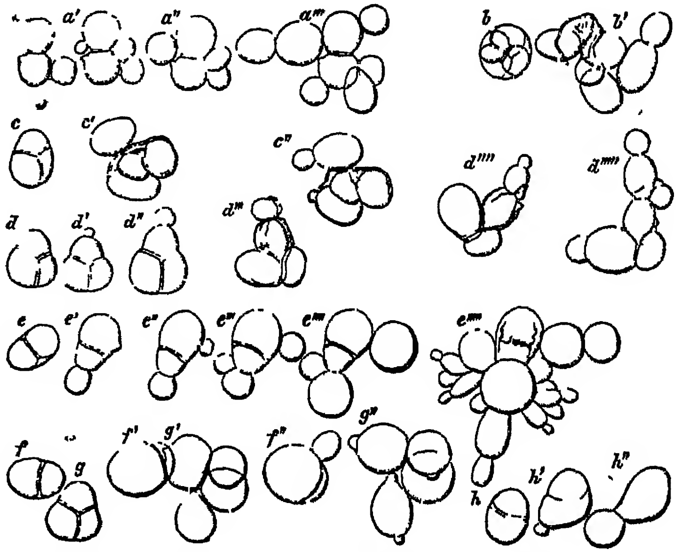


Fig 54—Budding of the spores in *Saccharomyces cerevisiæ I* (after Hansen)—*a*, Three spores without the wall of mother-cell, *b*, cell with four spores, *b'*, the wall of mother-cell is ruptured, *c*, cell with four spores, three of which are visible, *c'* and *c''* shows the ruptured wall of mother-cell, *d*, cell with three spores, *b'''*, the ruptured wall of mother-cell, *e-e'''''*, development of a very strong colony; *f-h*, other forms of development; h'', the wall between the two spores has disappeared

Budding can occur at any point on the surface of the swollen spores, it usually takes place after the wall of the mother-cell has been ruptured or absorbed, but it also occasionally takes place within the mother-cell After the buds have formed, the spores may remain connected, or they may soon break away from each other

Certain spores display a very remarkable behaviour (see Fig. 54 *e-e'''''* and *h-h''*), the absorption of the wall separating two neighbouring spores causing them to fuse together It is possible

that the biological significance of this phenomenon lies in the fact that the spores may thus have a greater chance of forming buds under unfavourable conditions One spore plays the part of a parasite to the other The union of the walls—described above—is, perhaps, the beginning of the process

A similar fusion of spores was observed by Hansen in the case of a wine yeast (*Johannisberg II*) He placed spore-forming cells in a shallow layer of wort In the course of a few hours they swelled up and burst the mother-cells They were then transferred to a shallow layer in a saturated aqueous solution of calcium sulphate at 25° C Under these conditions no budding took place, but several spores fused together and formed new endospores

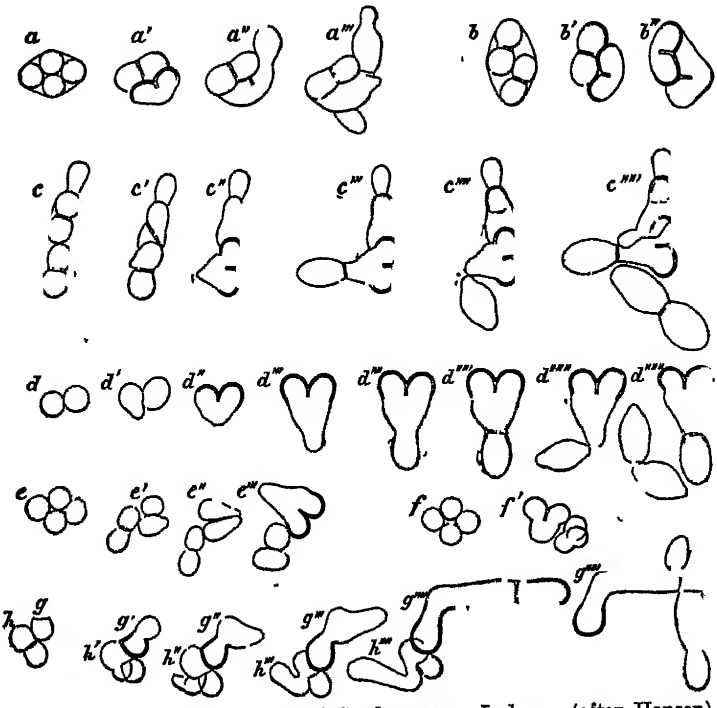


Fig 55 —Germination of the spores of *Saccharomyces Ludwigii* (after Hansen) —*a-c*, Gypsum-block cultures twelve days old, *d-h*, a similar culture six weeks old

Guilliermond found that in *S Saturnus* a similar fusion of spores takes place, and the same observations were made by Marchand in *S Pastorianus II and III*, *S ellips I and II*, *Johannisberg I*, *S vini Munzii*, *S. Willianus*, and *S Bayanus.*

On the surface of the two swollen spores a beak-shaped projection is observed, these are then seen to meet and form an anastomosis, the walls being broken up at the top A heart-shaped zygospore is thus formed, which will increase in size, the

conjugating part gradually widening The nuclei then merge into each other, and the bud-formation takes place at different points, most frequently from the conjugating channel.

In this connection we may mention the fusion (copulation) of vegetative cells observed by Schionning, Guilliermond, and Barker in the case of *S octosporus, Pombe, mellacei, and Zygosaccharomyces* Further delails are given in the systematic description of these species

S Ludwigii forms a second and very different type (Figs 55, 56), where germination does not take place through budding,

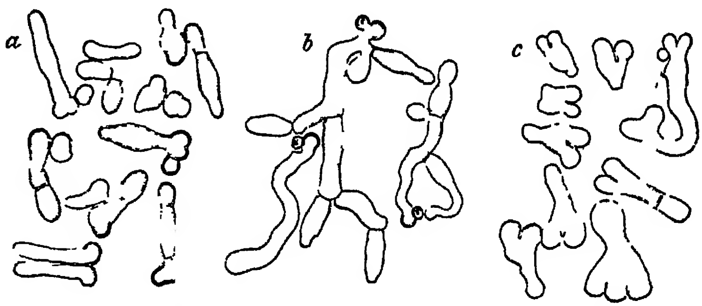


Fig 56 —*Saccharomyces Ludwigii* (after Hansen) —Germinating spores from old gypsum-block cultures, *a* and *b*, each has developed a germ-filament, *c*, shows different forms produced by fusion

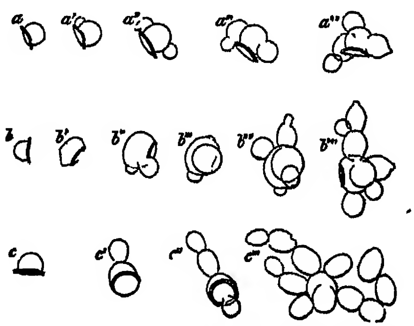


Fig 57 —Germination of spores of *Sacchromyces anomalus* (after Hansen)

but through a germinal tube, called a promycelium Two such germinal tubes frequently fuse together, and the propagation of yeast cells takes place through division and not through budding, after the formation of a clearly defined septum Similarly these yeast cells produce new cells In this case, unlike the first type, it is not the spores, but the new formations springing from them that fuse together Guilliermond observed such a fusion of germinal tubes in spores which were derived from different mother cells.

In older spores this curious fusion is more uncommon (Fig

56) A few germ-filaments develop into a branched mycelium (group *b*)

The spores of *S anomalus* (Fig 57) have a remarkable shape similar to those of *Endomyces decipiens.* They are almost hemispherical with a rim round the base

During germination the spores swell and the projecting rims may either remain or disappear Buds then crop out at different points on the surface of the spore

One object of Hansen's work was to determine to what extent the formation of spores was influenced by different temperatures, with a view to ascertaining whether the various species behave alike, or whether it might not be possible in this way to discover characteristics It was necessary to determine :—First, the limits of temperature—*i e*, the highest and lowest temperatures at which spores can be formed, secondly, the optimum temperature —*i e.*, the temperature at which spores appear most rapidly, and, thirdly, the relation between the intermediate temperatures.

To determine the time required, the moment was registered at which the cells showed distinct indications of spores It is not possible to make use of ripe spores in these determinations, since no criterion exists for complete ripeness

The results obtained by Hansen are as follows :—

The formation of spores takes place slowly at low temperatures, more rapidly as the temperature rises, until a point is passed at which their development is retarded and finally ceases

The lowest limit of temperature for the six species first investigated was found to be 0·5°-3° C, and the highest limit 37·5° C. Hansen also determined the intermediate temperature and time relations for the six species, and found that when these two values are graphically represented, with the degrees of temperature as abscissæ, and the time intervals as ordinates, the curves are almost identical for the six species They sink from the ordinates of the lowest temperature towards the axes of the abscissæ, and then rise. At the same time, however, these curves indicate that the cardinal points determined, more especially for the highest and lowest temperatures, give characteristic distinctions for the different species—*i e*, that the limits of temperature within which the formation of spores can take place differ for the various species (see systematic description)

In a course of years a number of investigators have carried out similar researches, including Holm, Will, Aderhold, Kayser, Seyffert, Marx, Schionning, and the author.

The following observations were made regarding the time required for the appearance of the first indications of spores in the six species maintained at the same temperature At the highest temperature thirty hours were required for the development of each species, at 25° there was again no great difference

in the time required, at the lower temperature, however, marked differences occurred Thus, in the case of *S cerevisiæ I.*, the first indications of spore-formation appear at 11·5° C after ten days, but in the case of *S Pastorianus II*, they appear within seventy-seven hours

In all such determinations a considerable influence is exerted by the state of the cells, and the results vary with the temperature at which they have been grown, with their age, vigour, etc (compare section on Variation of yeast cells) It follows that the composition of the nutrient fluid also exercises an influence Thus in methodical, comparative experiments of this nature, it is a necessary condition that the previous cultivation of the cells should always be carried out in the same manner If these external conditions are varied, the limits for the reactions of the species must be determined in each case.

By these experiments Hansen has established an important character for the determination of the Saccharomycetes It is also of great interest to note that spore-formation has a lower temperature maximum than budding, but a higher temperature minimum; in other words, spore-formation takes place within a narrower range of temperature than budding

The method given below for the practical analysis of low brewery yeast was based by Hansen on the temperature curves for the development of spores Thus, it was found that at certain temperatures the species employed in the brewery, the culture yeasts, develop their spores later than the wild yeasts, several species of which occur as disease germs in the brewery It is also important to note that the structure of the spores in these two groups is usually different The young spore of culture yeast has a distinct wall or membrane, the contents are not homogeneous, but are granular, and exhibit vacuoles In the case of wild yeast, on the other hand, the wall of the young spore is usually indistinct, the contents are homogeneous and strongly refractive It should also be added that the spores of culture yeasts are usually larger than those of wild yeasts

1. For the continuous daily control of low brewery yeast, as regards contamination with wild yeast, the following very convenient method is made use of:—At the conclusion of the primary fermentation, a small sample of the liquid is transferred from the fermenting vessel to a sterilised flask, this is set aside for some hours until the yeast has settled to the bottom, when the sediment is transferred to a gypsum block It is then placed in a thermostat at a temperature of either 25° C. or 15° C

It was shown that the species of culture yeasts employed in low-fermentation breweries can be divided into two groups This has subsequently been confirmed by the elaborate investigations of Holm and Poulsen. At 25° C, one group yields spores at a

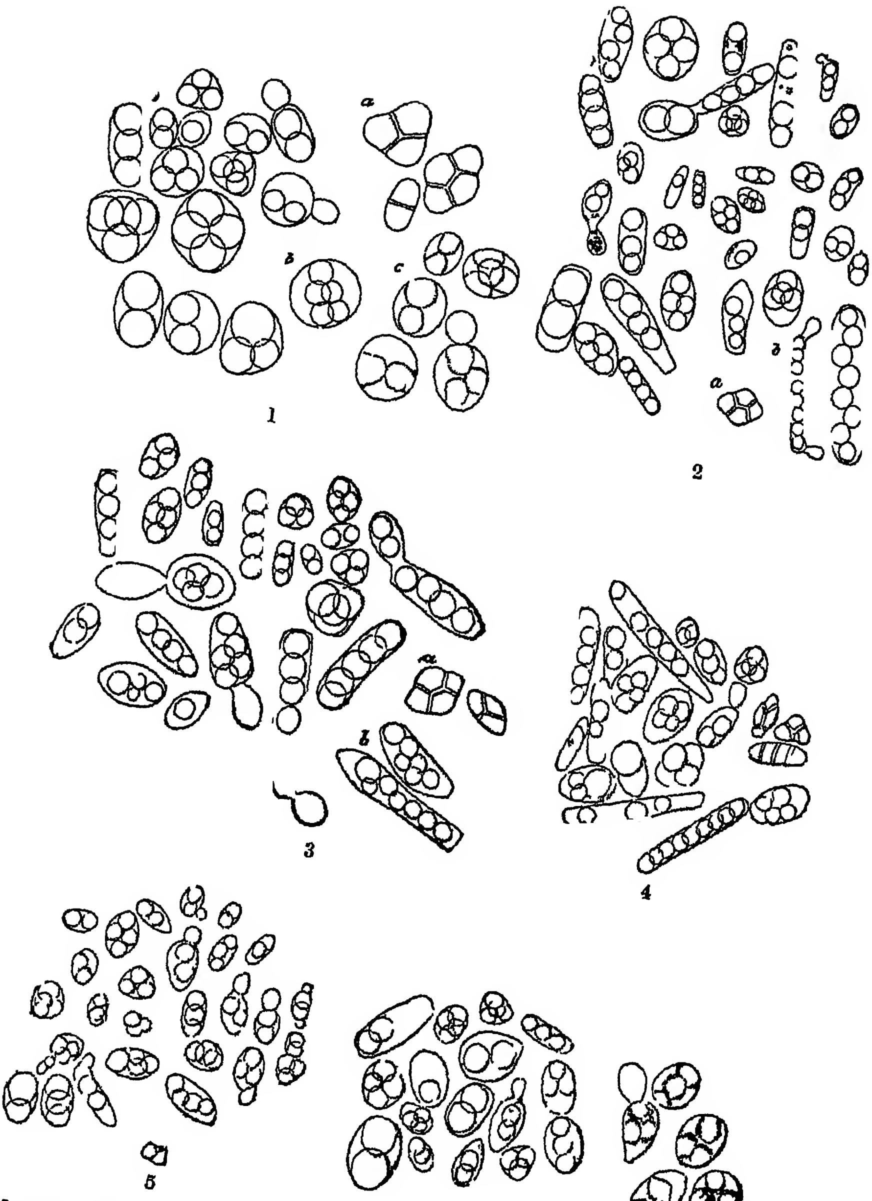


Fig 58.—*Saccharomyces* with ascospores (after Hansen).—1, *Sacch. cerevisiæ I*; 2, *Sacch. Pastorianus I*, 3, *Sacch Past II*, 4, *Sacch. Past III*; 5, *Sacch, ellipsoideus I*, 6, *Sacch ellips. II*, *a*, cells with partition-wall formation, *b*, cells containing a larger number of spores than usual, *c*, cells showing distinct rudiments of spores

later period than wild yeast, the other group produces spores in about the same time as wild yeast, but at a temperature of 15° C. the cells of wild yeast show spore-formation much sooner than the cells of either group of culture yeasts.

The cultures maintained at 25° C are examined after an interval of forty hours, and those maintained at 15° C. after an interval of three days

The author has shown that high brewery yeasts can be analysed in a similar manner In the case of some species, however, the analysis is best made at 10°-12° C, because a well-marked difference of time between the beginning of spore-formation in culture yeast on the one hand and wild yeast on the other can only be observed at this temperature

According to the author's researches, distillers' yeast may be analysed in the same way Lower temperatures are to be preferred for this analysis. Often, however, the investigation into the construction of the spore in the selected yeast-type must form the chief part of the analysis, the difference of time for spore-formation in culture yeast and wild yeast frequently proving inadequate

Aderhold has established the fact that wine yeast, like beer yeast, may be analysed by Hansen's method

By means of experiments undertaken to determine to what extent Hansen's analytical method can be relied on for technical purposes in low-fermentation breweries, Holm and Poulsen concluded that a very small admixture of wild yeast, about $\frac{1}{200}$th of the entire mass (Carlsberg bottom-yeast No 1), can be detected with certainty Hansen's previous researches had shown that when the two species, *Sacch Pastorianus III.* and *Sacch. ellipsoideus II*—which are capable of producing yeast-turbidity in beer—are present to the extent of only 1 part in 41 of the pitching yeast, the disease is not developed, provided that the normal conditions of fermentation and storage have been maintained Further, *Sacch Pastorianus I*, which imparts to beer a disagreeable odour and an unpleasant bitter taste, can scarcely exert its injurious influence under the same conditions when the admixture of this yeast amounts to less than 1 part in 22 of the pitching yeast Consequently, Hansen's method for the analysis of yeast by means of ascospore formation is sufficient to establish its purity *

When the object of the analysis is to characterise the different species present in the sample with greater accuracy, a number of

* It is obvious that such an analysis from the vat does not enable direct conclusions to be drawn regarding the predominant biological conditions existing during secondary fermentation in the cask If, for instance, the beer is run off with a very small quantity of yeast, even if the infection is a small one, the wild yeast will chiefly be found floating in the liquid, and will be carried over into the cask, whilst the greater part of the culture yeasts will have sunk to the bottom of the vat

cells are isolated by fractionation, and each of the growths obtained is separately examined

In an investigation of bottom yeast in the different stages of the primary fermentation, published by Hansen in 1883, it was shown that the young cells of wild yeast are present in largest amount during the last stages of primary fermentation and in the upper layers of the liquid The samples taken from the fermenting vessel for the analysis of yeast must, therefore, be taken during the last few days of the primary fermentation If a dried or partially dried sample of yeast is to be examined, it must be first transferred to wort, and one or more fermentations must be completely carried out with it

The rule that wild yeasts develop only in the more advanced stages of fermentation applies also to top-fermentation yeast as used in breweries This was shown by numerous analyses of beer from Danish, English, French, and German breweries carried out in the author's laboratory As is well known, it was this very appearance of wild yeast in English top-fermentation breweries which gave rise to the erroneous view that such species are necessary for conducting a normal secondary fermentation.

2. The analysis of the yeast in the propagating apparatus, which must be absolutely pure, is carried out as follows —At the conclusion of fermentation, samples are withdrawn, with every precaution, into Pasteur flasks or into the Hansen flasks employed for sending out yeast samples, from these, small quantities are introduced into flasks containing neutral or slightly alkaline yeast water or yeast-water dextrose, and maintained at a temperature of 25° C, the object being to test the yeast for bacteria The remainder is set aside to allow the yeast to settle, the beer is decanted, and an average sample of the sediment is introduced into a cane-sugar solution containing 1 to 4 per cent. of tartaric acid After three or four cultivations in such a solution it is further cultivated a few times in beer-wort, and then tested for spore-formation. The smallest traces of wild yeast in the apparatus are brought into a state of vigorous development by this treatment *

I. SACCHAROMYCES.

The name *Saccharomyces* is used to distinguish budding fungi with endogenous spore-formation The great majority of species are only known in this form, but a few can develop a mycelium. In the case of one particular group of *Schizosaccharomyces* division of the cell takes place instead of budding, exactly in the same way as with certain of the mould fungi

* It is evident that this method is not available for the analysis of ordinary yeast, because the cultivation in the tartaric solution will cause the wild yeast cells to increase very considerably in number, and consequently render it impossible for the analyst to judge of the degree of contamination

In addition to these fungi many other kinds of budding species occur in nature which do not display endogenous spore-formation Thanks to investigations by de Bary, Zopf, Brefeld, and others, it is now known that certain of these are developed from the higher fungi *Ustilagineæ* (smut-fungi), *Basidiomycetes*, etc

A glance at the following figures shows that the *Saccharomycetes* may develop mycelial cells in their films Thus cultures of *S. Marxianus* may occur with typical branched mycelium Such formations may probably be regarded as tending to show that if these fungi are afforded more favourable conditions of development in nature than those obtaining when they are artificially cultivated in a laboratory, they are likely to develop as typical moulds The following observations of the author appears to favour this view —On dried grapes, growths of *Dematium*-like moulds have been observed with a rich formation of spores (see Fig 45) * If such growths are cultivated either in or upon a substratum in flasks, their spores develop only budding cells with endogenous spore-formation In the same way vigorous growths of mould have been found on slices of Agave stems from Mexico, which at first suggest *Monilia*, and give a strong formation of spores By cultivation in nutritive liquids and on gelatine only *Saccharomyces* cells are produced On saccharine material received from Jamaica, growths of moulds were found resembling *Oidium*, but the cells also exhibit spore-formation, and by further development of the mould in and upon sterile substrata nothing but a growth of *Schizosaccharomyces* is obtained, and no mycelium †

In all three cases such substrata were utilised as had otherwise proved favourable for the growth of moulds But it was impossible, under laboratory conditions, to reproduce the natural conditions which favour the formation of these *Dematium*, *Monilia*, and *Oidium*-like fungi.

Further investigation will determine how far such conditions are to be found in nature These observations, at any rate, show that there are cases where the natural conditions allow of a development which cannot be substantiated by artificial conditions in the laboratory, and the conclusion appears to be warranted that other fungi, including higher fungi, may behave in the same way, like the *Ustilagineæ* and other forms which regularly reproduce budding growths incapable of forming endospores An isolated example of the development of *Saccharomyces* cells from a fungus, *Glœosporium*, belonging to a higher system, has been recorded by Vialla and Pacottet As stated above, a development of budding cells from the conidia of *Aspergillus Oryzæ* was

* The fungi do not possess the characteristic coloured and thick-walled resting cells of *Dematium pullulans*

† Lepeschkin observed a similar weak formation of mycelium from individual cells of *S. mellacei*.

observed in the author's laboratory (1895) In later researches the endogenous spore-formation first observed could not be reproduced. A development of budding cells from the gemmæ of the mycelium of this species was noticed in 1922 by Zikes. The precise conditions under which this and the former transformation are produced are not yet known

The basis of a scientific system of classification was suggested by Hansen in 1904 as follows —

Family—Saccharomycetes.

Budding fungi with endospores and vigorous formation of yeast cells Typical mycelium occurs only in few cases Every cell may occur as the mother-cell of a spore Spores unicellular. Number of spores in each mother-cell usually from one to four, seldom up to twelve

A. True Saccharomycetes

1st Group

The cells immediately form sedimentary yeast in saccharine nutritive liquids, and only at a much later stage form a film with slimy growth and without inclusion of air. Spores smooth, round, or oval, with one or two membranes Germination by budding or by the formation of germinal tubes (promycelium). All, or at any rate the great majority of this group bring about alcoholic fermentation

Genus I —*Saccharomyces* Meyen

The spores, provided with one membrane, germinate by budding. In addition to formation of the yeast cells a few give mycelium with distinct transverse walls.

(To this genus belong the culture yeasts and the great majority of wild yeasts)

Genus II —*Zygosaccharomyces* Barker

Distinguished by the copulation of cells In other respects identical with the preceding genus

Genus III.—*Saccharomycodes* E. C Hansen

By germination the spores possessing one membrane form a promycelium From these, as well as from the vegetative cells, budding takes place with incomplete separation. Formation of mycelium with distinct transverse walls.

Genus IV —*Saccharomycopsis* Schionning.

The spores possess two membranes, otherwise the characters, so far as they are known, are identical with those of *Saccharomyces*

2nd Group

The cells immediately form a film in saccharine nutritive liquid, which is dry and opaque on account of the inclusion of air, and can readily be distinguished from the film-formation of the first group The spores are hemispherical, angular, hat-shaped or lemon-shaped, in the last two cases provided with a distinct projecting rim, otherwise smooth. They have only one membrane, germination takes place by budding. The majority of species are distinguished by the formation of esters, a few do not bring about fermentation

Genus V —*Pichia* E C Hansen

The spores hemispherical or irregular and angular Very feeble or no fermentation

Genus VI —*Willia* E C. Hansen

Spores hat-shaped or lemon-shaped with distinctly projecting rims The majority produce esters vigorously, a few produce no fermentation

B Doubtful Saccharomycetes

(*Monospora* *Nematospora*)

Schizosaccharomyces

Rectangular or rounded cells dividing by partition-walls and frequently developing a separated mycelium Spores are formed generally after copulation and four in number These fungi look like spore-forming bacteria

1 The Species used Industrially (Culture Yeasts)

(*a*) **Brewery Yeasts.**

According to the physical phenomena of fermentation, a distinction is made between low- and high-fermentation yeasts, both in the brewery and elsewhere The low-fermentation yeasts gradually collect during fermentation to form a deposit in the fermenting liquid, whereas the top-fermentation yeasts, in the normal course of fermentation, partly form a layer on the surface of the liquid, differing in character and thickness according to the race, and partly form a deposit. The two kinds of yeasts in the brewery impart a different character to the fermented liquor. This has been established by parallel experiments with wort of

identical composition. The two groups of yeast may, therefore, be said to exhibit a different form of chemical activity. Bau has proved that most of the known species of low-fermentation yeasts ferment melibiose, whilst some of the top yeasts are incapable of fermenting it. According to Bau and Fischer, melibiase, the enzyme that ferments melibiose, could only be detected in low-fermentation yeasts, and not in those top yeasts that are unable to ferment melibiose.

According to Hansen's work (referred to in the section on Variation), individuals may occur in old cultures of bottom yeasts which exhibit top-fermentation phenomena, and similarly, although in smaller numbers, individuals in top yeasts which behave like bottom yeasts.

After Hansen had introduced a pure culture of bottom yeast into the Carlsberg brewery in Copenhagen it was possible to discover how extensive and how deep-seated the differences are which distinguish the various bottom-fermentation brewery yeasts. With this object in view the writer undertook a long series of comparative experiments with pure cultures of top and bottom yeasts from every part of the world, noting in particular the degree of fermentation, the clarifying power of the liquor, the physical phenomena of fermentation, and the stability of the fermented liquor. As early as 1886, in the first edition of this book, he set forth a classification of typical species or races, the correctness of which has been confirmed by subsequent workers in this field.

A. Bottom-fermentation Species

1. Species which clarify very quickly and give a feeble fermentation, the beer holds a strong head. The beer, if kept long, is liable to yeast-turbidity. Such yeasts are only suitable for draught beer.
2. Species which clarify fairly quickly and do not give a vigorous fermentation, the beer holds a strong head; high foam. the yeast settles to a firm layer in the fermenting vessel. The beer is not particularly stable as regards yeast-turbidity. These yeasts are suitable for draught beer and some for lager beer.
3. Species which clarify slowly and attenuate more strongly. The beer is very stable to yeast-turbidity. These yeasts are suitable for lager beer, and especially for export beers.

B. Top-fermentation Species

1. Species which attenuate slightly and clarify quickly. The beer has a sweet taste.
2. Species which attenuate strongly and clarify quickly. Taste of beer more pronounced.

3 Species which attenuate strongly and often clarify slowly. The beer is stable to yeast-turbidity

By far the greater number of high-fermentation yeasts examined in this respect are able to carry through a secondary fermentation In class 2, and especially 3, the secondary fermentation is very vigorous and long continued

Before the results of these comparative experiments had been published, both Hansen and the author had had the opportunity, as will be seen, of demonstrating that many of the species so characterised appeared as strongly marked types when applied in the form of mass cultures in practice, and that both in the above and other respects typical differences made their appearance between the individual races or species which found application as culture yeasts The experience gained during the years that have since elapsed goes to prove that by a methodical selection of a race, an element of certainty is introduced into the fermenting conditions, which was impossible when a mixed yeast of unknown composition was employed

In 1884 Hansen made the following pronouncement :—

"We find by closer investigation that differences exist amongst the kinds of yeast which must be described as good from the standpoint of the brewer Thus, under similar conditions, some give a quicker and more complete clarification in the primary fermentation and a more feeble attenuation than others. Again, differences are found in respect to flavour If my method is followed, it is possible, nevertheless, to select with care and quite methodically, that species which is best suited for the particular work. This phase of the question has been practically solved at Old Carlsberg, where a yeast has been selected, in addition to that previously described, which is better suited for making lager beer, whilst the former is better for export beer Where the fermentation industry formerly groped blindfold, and everything was a matter of guess work, a path has now been opened to an exact technique"

Hansen is here referring to the two races of yeast that were first isolated and described

To what extent individual types display a pronounced character in their practical application depends largely on the nature of the treatment Thus the degree of fermentation is determined both by the composition of the liquid and by the other conditions of fermentation A race which gives a vigorous fermentation, can obviously only display this property under certain conditions On the other hand, typical characters exist which may become noticeable under very different external conditions Thus it was shown by the author, in the earliest stages of the development of this important reform, that top-fermentation species from the brewery, which have a definite influence on the

odour and flavour of the fermented liquor, can be recognised by this fact when they are used in breweries in distant countries, where both the raw materials and the methods of working may be entirely different The same applies when such species are introduced in absolutely pure cultures, which, beginning on the small scale with one or two litres of thin yeast liquor, are propagated by degrees in brewery wort and thus adapted to it

The two first races obtained as pure cultures, referred to by Hansen in the above quotation, were —*Carlsberg No* 1, a yeast applied for many years in the Carlsberg brewery in Copenhagen, and *Carlsberg No* 2, which was introduced from a German into a Copenhagen brewery, the fermentation being under the control of the author After he had drawn attention to the remarkable fermentation phenomena observed with this yeast, which differ widely from that of *Carlsberg No* 1, it was introduced into the Carlsberg brewery and isolated as a pure culture by Hansen and the author

In 1885 the author had the opportunity of answering the question whether different races or species of *S cerevisiæ* exist, the answer being based upon his own botanical and biological investigations of these two species

The *Race I* chiefly exhibits slightly elongated cells, amongst which somewhat smaller pointed individuals distinguished by granular contents are not infrequently found If the yeast is taken from the fermenting vessel, washed with water, and placed for a short time on ice, it will be observed that the whole cell content rapidly changes to a granular structure, and if maintained under these conditions for several days, it will be found that the number of dead cells rapidly increases. The *Race II.* behaves in quite a different way. The cells are short and oval, or almost spherical, under normal conditions in the fermenting vessel, only a few weakened individuals are observed, and in a washed condition the cells retain their clear or slightly granular contents for a long time, very few dead cells are observed even after long preservation in this condition.

If each of the growths is placed on moist gypsum blocks, maintained at the same temperature, and their further development observed from day to day, it will be seen that the two races behave quite differently, assuming that the temperature lies within the limits for the growth of spores *Race II.* forms fully ripe spores at a time when *Race I* does not show a trace of these organs of propagation

The following distinctions are of value in practice in determining the two races —

In order to obtain the normal course of the primary fermentation it is essential that *Race I* should be introduced at a somewhat higher temperature (7 5° C.) than *Race II* Larger

quantities of *I* than of *II* must be used for pitching, in the proportion of 66 to 58 The time of setting and of frothing naturally differs Both phenomena appear to occur somewhat earlier with *Race II.* than with *Race I*

The nature of the frothing and the coating of yeast differ greatly *II.* gives a strong high head and a dense coherent cover, *I* a low head, and the liquid often shows bald patches. Moreover, *Race I* gives a very lasting fermentation, and, as a consequence, a slower clarification than *Race II*, which when pure gives a particularly bright clarification The sedimentary yeast in the vat lies more compactly, and the colour of *II* is somewhat lighter than that of *I*.

The attenuation during the primary and secondary fermentation with normal wort and in the same brewery is stronger with *Race I.* than *II*

With regard to the finished beer, similar differences are noted, particularly regarding flavour and resistance to turbidity Most experts prefer *II.* for flavour, but some difference of opinion exists It is otherwise regarding the stability of the beer, especially with regard to yeast turbidity In this respect the difference is very marked *I* gives a quite exceptionally stable beer, and is specially suitable for export beer, which when fermented with this yeast remains unaltered for about a month without any further treatment, and by mild pasteurisation is rendered stable for much longer periods. *Race II*, although it displays much finer phenomena during the primary fermentation, is unable to produce completely stable beer (about ten days in bottles at room temperature), and it is also noteworthy that this race is much less resistant to wild yeast than *I*. On account of the rapid clarification and quick fermentation of the liquor this race is adapted for beers which are to be stored for a short time, and are to be consumed immediately.

In general, it may be stated that the whole of the differences indicated have been observed for years in different breweries, and that they are so sharply defined that every brewer could at once distinguish the two yeasts with certainty when they have been put into the fermenting vessel, and could foretell the nature of primary and secondary fermentation. In fact, no one could be in doubt that we are dealing with two truly distinct races or species.

In the detailed descriptions of these two races, published by Hansen in 1888 (which might equally well have appeared before the author's publications, as will be seen from the preceding historical description), the characteristic distinctions between these two species are further emphasised. Amongst other observations, reference is made to the giant cells, remarkable and abnormal large round cells which suggest the cells of *Mucor* yeasts

In 1908 Hansen described further typical differences, and gave the species the names *S Carlsbergensis* and *S Monacensis*

S. Carlsbergensis (= *No* 1) has temperature limits for budding in wort at about 33·5° C and 0° C At the maximum temperature the cells are considerably larger than at the outset, but have approximately the same shape, giant cells are numerous At the minimum and up to 9° C many of the cells assume the sausage-shape, and form large mycelial colonies In the films elliptical and round cells principally occur The giant colonies are rosette-shaped with a depression, and less frequently a distinct knot in the middle, with concentric rings and radial streamers, they have a smooth or scaly surface and wavy outline The colonies in the usual plate-cultures are built up as small pin heads with a light greyish-yellow and waxy appearance. This yeast assimilates glucose, maltose, and saccharose dissolved in yeast-water, as also in solutions of asparagine and peptone with nutritive salts (Klocker).

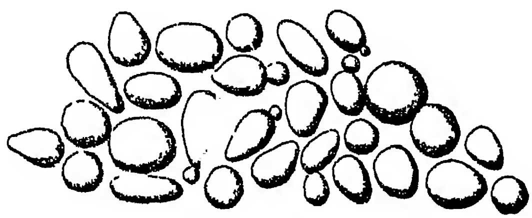


Fig 59 — Carlsberg low-fermentation yeast No. 1 (after Hansen)

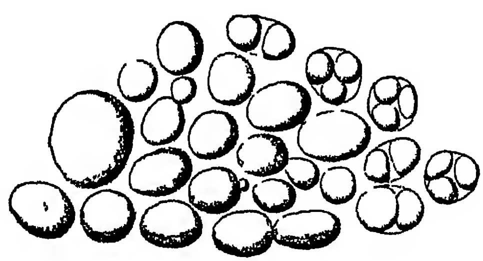


Fig. 60 —Carlsberg low-fermentation yeast No 2—a few cells with spores (after Hansen).

S. Monacensis (= *No* 2) has temperature limits for budding at about 33° and 1°. At the maximum the cells are larger, and especially longer, than at the outset, and at the minimum and up to 9° C, in contrast to the former species, it develops colonies consisting principally of spherical and elliptical cells. In dextrose-yeast-water the giant cells may assume huge dimensions In the film the cells are spherical and elliptical The giant colonies

and the small colonies agree in appearance with those of the former species *

As an admirable example of the application of Hansen's biological methods to the differentiation of yeast species, we give the comparison of four low-fermentation brewery yeasts carried out by Will This, again, emphasises the fact that, within this group, species occur just as clearly distinguished as in those groups of Saccharomycetes which have not yet found an industrial application Will began his characterisation by adopting the classification of brewery-yeast types published by the writer in 1886 He classed *Races* 93 and 2 as high-fermenting, *Race* 7 as a low-fermenting type, and *Race* 6 as a yeast of intermediate fermentation

The four yeasts can be distinguished as follows —

Race 2 has large roundish or oval cells, the colonies on gelatine are spherical or lenticular, the spores are formed easily and freely, spore-formation takes place between 31° and 11°, the optimum is 25°-26°, film-formation occurs between 31° and 7°, very slow

Race 6, oval cells predominate, but the species has a great tendency to form sausage-shaped cells, colonies on gelatine are spherical or lenticular, the spores form easily and freely; spore-formation occurs between 31° and 11° C., the optimum is 28°, film-formation between 31° and 7°, occurring later than with *Race* 7

The cells of *Race* 93 are typically oval with a tendency to assume a roundish shape The colonies in gelatine are spherical or lenticular, spores are freely and easily formed, spore-formation occurs between 30° and 10° C, optimum 28°, film-formation between 31° and 4°, very feeble and slow, resting cells occur freely in the film

Race 7 has oval cells which closely approach the spherical shape, giant cells regularly occur, and at the end of the fermentation large budding colonies with small oval cells frequently occur, the young colonies on gelatine are irregular with a marked wavy and fringed outline, the species develops spores with great difficulty, spore-formation occurs between 30° and 13°, optimum 25° to 26°, film-formation between 28° and 4°, appears earlier than with the other species, resting cells are to be found only in small numbers in the film

In the case of strains Nos 2, 6, and 93, the upper temperature limit of bud-formation in wort is at 36° C, in No. 7 at 34° C;

* Further examples of the formative power of temperature will be found in the following descriptions of species As pointed out by Zikes, many species behave like *S Carlsbergensis*, at low temperatures the cells are lengthened or sausage-shaped, and mostly remain grouped together in budding associations, while at higher temperatures the same species will present shorter, globular, or egg-shaped cells, which are soon disunited.

the lower limit being in all four about 1° C. It deserves notice that these four bottom-yeasts at the extreme temperatures will form large budding-associations of thick-walled cells.

P. Lindner distinguished two species of low-fermentation yeast in 1889, which he called "Saaz" (weakly fermenting) and "Frohberg" (strongly fermenting). These names have been adopted in the literature as a description of weakly and strongly fermenting yeast types in general.

A thorough description of two low-fermentation yeasts of the Frohberg type (D and K) has been given by Schönfeld and Rommel. D gives longish, almost sausage-shaped cells, K predominantly spherical and oval cells. D is more inclined to form spores than K. In hanging drops, differences in the shape and size of the cells can be remarked. K forms budding colonies more rapidly and in greater number. In the growth of giant colonies similar differences have been observed. The content of albumen, the percentage of ash and phosphoric acid are higher in K than in D, and K has a higher specific gravity than D. Auto-digestion occurs more rapidly with K. K has a higher "raising power," and is more sensitive to high temperatures. Fermentation sets in more rapidly with K. At temperatures above 30° the fermentative activity of K yeast is weakened to a much greater extent than that of D. The film growth of K is capable of fermenting more carbohydrate than the sedimentary yeast, whilst with D the difference is unimportant. In the brewery, K ferments 10 per cent. higher than D in the fermenting vessel, and gives a lighter coloured beer. The final fermentation is identical with the two species.

Schönfeld defines two types of yeast with the names of "Bruchhefe" (Breach Yeast) and "Staubhefe" (Dusty Yeast), the peculiarities shown in the course of fermentation being recorded in these names. They occur under definite external conditions. Schönfeld considers that the former type chiefly arises in breweries using more or less marked calcium carbonate water, but dusty yeast in such as employ water containing a large amount of calcium sulphate. When grown in the same nutrient liquid, the breach yeast is believed to have a higher percentage of albumen and ash than the other type.

In 1883-85 very detailed researches were carried out by the author in elaborating the principle laid down by Hansen, and introduced in the Carlsberg brewery, namely, the application of methodically selected pure cultures derived from a single cell. The experiments were carried out with a view to securing practical conditions, and the results obtained in the laboratory were applied on a large scale in breweries in many European countries. The reform found acceptance with prominent fermentation technologists (especially Thausing, Lintner, and Aubry) in the early stages, and

it was gradually incorporated into the courses of all zymotechnological institutes

Saccharomyces cerevisiæ or Saccharomyces cerevisiæ I. Hansen.

This species, described in 1883, is an old English top-fermentation yeast which is in use in London and Edinburgh breweries

The young growth of sedimentary yeast developed in wort consists essentially of large round and oval cells, truly elongated cells do not occur under these conditions

Ascospore-formation —

At 37·5° C	no ascospores are developed	
36-37	the first indications are seen after	29 hours.
35	,, ,,	25 ,,
33·5	,, ,,	23 ,,
30	,, ,,	20 ,,
25	,, ,,	23 ,,
23	,, ,,	27 ,,
17·5	,, ,,	50 ,,
16·5	,, ,,	65 ,,
11-12	,, ,,	10 days.
9	no ascospores are developed	

Wall of spores very distinct. Size of spores 2·5 to 6 μ.

Film-formation —

At 38° C.	no film-formation occurs	
33-34	feebly-developed film specks are seen after . . .	9-18 days.
26-28	,, ,,	7-11 ,,
20-22	,, ,,	7-10 ,,
13-15	,, ,,	15-30 ,,
6- 7	,, ,,	2- 3 months.
5	no film-formation occurs.	

Microscopical appearance of the cells in the films :—

At 20° to 34° C, colonies frequent; sausage-shaped and curiously formed cells occur.

At 15° to 6° C —The majority of the cells resemble the original; isolated abnormal forms occur

In old cultures of films all forms occur, including extremely elongated mycelial cells

The temperature limits for budding in wort are 40° C and 1° to 3° C. The species develops invertase and maltase; it ferments saccharose, maltose, and dextrose, but not lactose. It produces a vigorous fermentation in beer-wort, and assimilates glucose, maltose, and saccharose in yeast-water, and in asparagine and peptone solutions with nutritive salts.

The first series of pure top-fermentation species were prepared by the author in 1884 from material collected in many European countries, with the object of introducing such pure selected types, developed from single cells, into practice. It was soon seen that the typical differences between the species were much more pronounced than is the case with low-fermentation yeast It was found that one group of the species used in breweries had an extraordinary weak fermentative activity The fermentation ceased, under the conditions existing in the breweries, when 1 to 2 per cent of alcohol had been formed in beer-wort, the main mass of the yeast usually spread out over the surface of the liquid to form a coherent layer

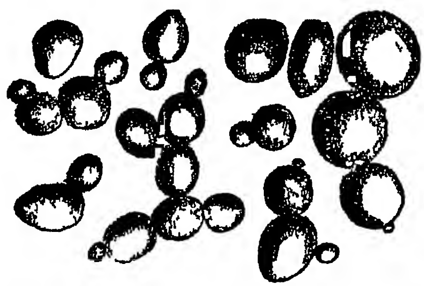


Fig 61 —*Saccharomyces cerevisiæ I* (Hansen) —Cell-forms of young sedimentary yeast (after Hansen)

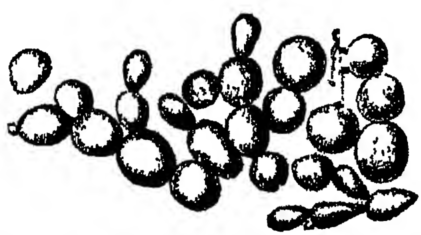


Fig. 62.—*Saccharomyces cerevisiæ I* (Hansen) —Film-forms at 15° to 6° C. (after Hansen)

Species of the second group behave quite differently. Under similar conditions fermentation can be carried on for a much longer time; clarification goes on slowly; and when the primary fermentation is at an end the beer is decanted from the yeast, which is largely sedimentary. A secondary fermentation takes place, the duration varying according to the species

As representatives of the first group, the species chiefly used in Danish breweries may be mentioned, and of the second group, many of those adopted in English breweries

The purely-cultivated Danish top-fermentation species fall into two distinct types, according to their chemical activity. The first impart a decidedly mild flavour; the fermentation is

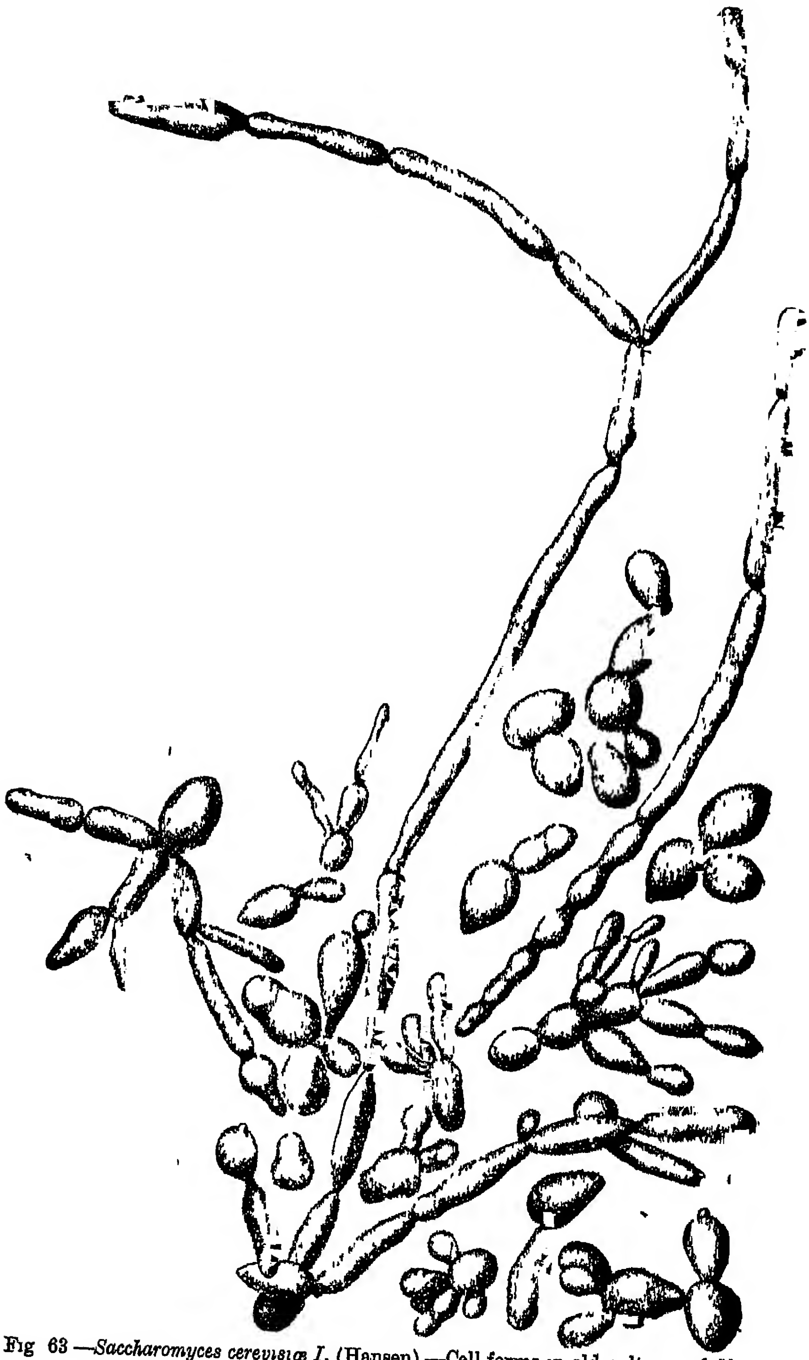


Fig 63 —*Saccharomyces cerevisiæ I.* (Hansen) —Cell-forms in old cultures of films. (after Hansen)

weak without noticeable secondary fermentation, and the layer of yeast forming on the surface of the liquid is loose and slimy. The second type gives a strongly pronounced flavour, the fermentation is stronger, with a tendency to secondary fermentation, and the layer of yeast has a dense consistency By long-continued use of both types the latter has proved to be more generally acceptable

The English species that have been subjected to examination, and have been proved to bring about a distinct secondary fermentation, exhibit a great variety of form and a varying construction of spores Many such differences have been recorded, both morphological and in relation to fermentation According to the observations of the author, the following facts have been established :—

The formation of cells at the different stages of alcoholic fermentation was determined by growths which were first kept for a long time in a 10 per cent sugar solution, then grown for several generations in beer-wort, and finally developed for twenty-four hours in Pasteur flasks at 25° C. The development of the films and their appearance to the naked eye were studied in cultures in Erlenmeyer flasks at room temperature (about 20° C) Cultures in Pasteur flasks at room temperature were used for determining the physical character of the sedimentary yeast. The fermentation experiments were carried out at room temperature in sterilised, hopped wort contained in tall cylindrical glasses covered with several layers of filter paper After the primary fermentation was completed, the liquids were poured into sterile flasks and allowed to stand at low temperatures The amount of alcohol was determined at the completion of the primary fermentation and again after the first fortnight of the secondary fermentation, and, lastly, after the following fortnight. The primary fermentation was interrupted when the appearance of the cells showed that the first vigorous development had ceased. In this comparison no attempt was made to ascertain the total quantity of alcohol produced by the species during primary and secondary fermentation the object was simply to institute a comparison

The flavour of the fermented liquor was recorded after the beer had undergone a secondary fermentation at a low temperature in flasks closed first with cotton-wool and afterwards with ground-glass stoppers

1. (*Fig*. 64, *a*, *and Fig*. 65, *a*)

The cells during fermentation are comparatively small, oval, and linked in chains , among them occur big, round and grotesque forms.

The yeast lies rather loose in the flask; if shaken it does not distribute itself equally in the wort, but separates into clots.]

Film-formation. After a lapse of 31 to 32 days a very thin film covers almost the whole surface of the liquid.

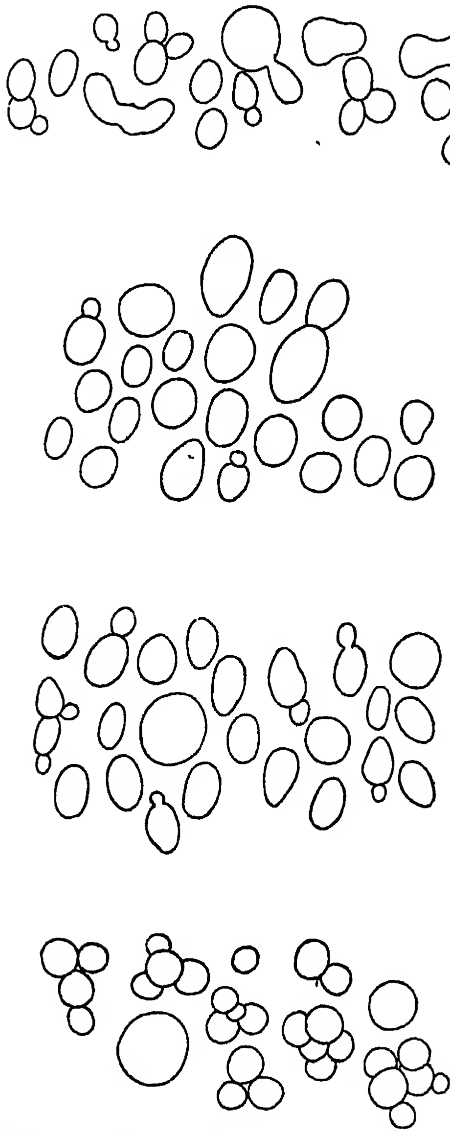
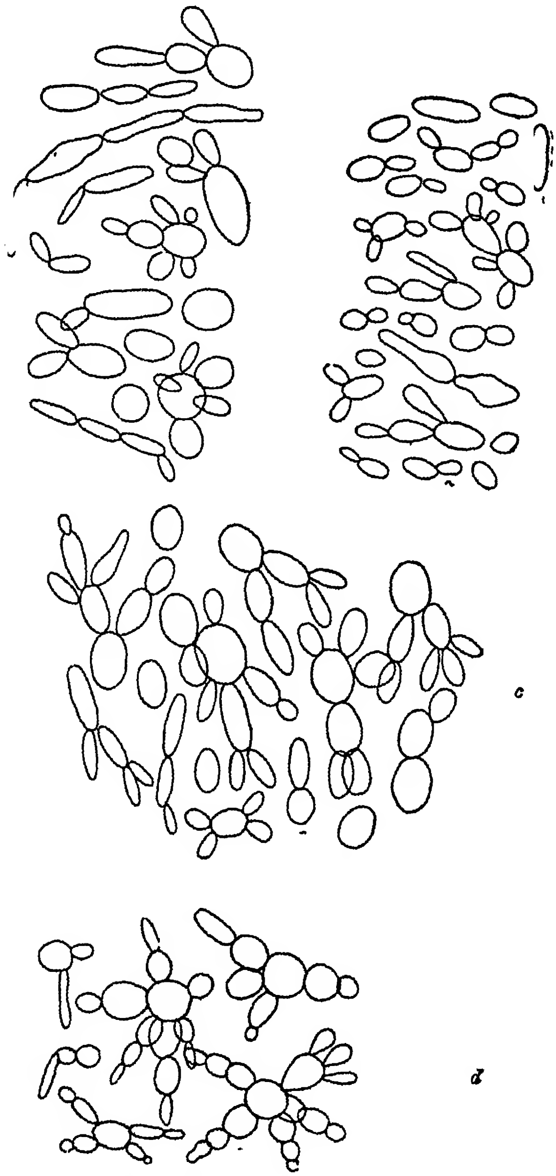


Fig. 64 —*a-d*, Young growths of English top-yeasts (Holm).

The cells of the film are of about the same size as those seen during the primary fermentation, some cells much elongated.

The spores, if developed at a low temperature, are small, full of vacuoles, and slightly granulated, as a rule, only one or two in each cell.



a b c d

Fig. 65.—*a-d*, Film-formations of English top-yeasts (Jorgensen)

At 11° to 12° C. a few spores make their appearance on the seventh day; at 25° C. abundant development of spores in forty hours.

When the principal fermentation was interrupted, the liquid contained 2·49 per cent by vol. of alcohol, during the two following periods (see above) 0 31 and 0 57 per cent by vol. were produced

Production of acid, after expulsion of CO_2, corresponds to 5 c c. of decinormal caustic soda solution

The fermented liquid has an agreeable smell and a fine aromatic taste.

2 *Fig* 64, *b*, *and Fig* 65, *b*.)

During fermentation most cells are free, medium-sized, round and oval, among them there occur round and oval giant cells

The yeast lies loose in the flask, if shaken slightly, it is distributed like a cloud throughout the liquid

Film-formation After 31 to 32 days, a few large patches.

The cells of the film are smaller than those seen during primary fermentation, ellipsoidal and slightly lengthened

The spores are big, if developed at a low temperature, formation of partition walls readily occurs

At 11° to 12° C very few spores occur on the seventh day; at 25° C rather abundant spore-formation in forty hours.

When the principal fermentation was interrupted, the liquid contained 2·3 per cent by vol of alcohol, in the two following periods 1 and 0 46 per cent by vol were formed.

Acid-production 6

Disagreeable smell and taste

3. (*Fig* 64, *c*, *and Fig* 65, *c*)

During fermentation the growth shows free cells and small chain-formations of oval forms, a few globular giant cells.

The yeast lies very compact in the flask, it partially rises in the liquid only when violently shaken

Film-formation In 31 to 32 days the growth forms a very thin film, which does not cover the entire surface of the liquid

Some of the cells of the film have the same size and shape as those seen during primary fermentation, others are slightly lengthened

If developed at a low temperature, the spores are of very varied size, with comparatively feeble refractivity, and without distinct vacuoles Partition-wall formations occur At 11° to 12° C., in seven days, only rudiments of spores appear; at 25° C, in forty hours, spores are very freely formed

When the principal fermentation was interrupted, the liquid contained 2·26 per cent by vol of alcohol, during the following two periods 0·79 and 0 00 per cent by vol were formed.

Acid-production 5·5

Disagreeable smell and taste

4. (*Fig* 64, *d, and Fig* 65, *d*)

During fermentation, colonies consisting of many small spherical cells occur, and among these spherical giant cells

The yeast lies loose in the flask, if slightly shaken, it is distributed like a cloud throughout the whole liquid

Film-formation After 31 to 32 days, only the merest trace.

The cells of the ring-growth occur in colonies, which sometimes contain upwards of a hundred cells, all derived from a single cell, the youngest growths are elongated and very narrow.

The spores, if developed at a low temperature, are small and vacuolised At 11° to 12° C, even after a fortnight, no spore-formation, at 25° C, for forty hours, a very scanty development of spores

When the principal fermentation was interrupted, the liquid contained 1 8 per cent by vol of alcohol, during the following two periods 1 and 0·82 per cent by vol were formed.

Acid-production 5 5.

Disagreeable smell and taste

5 (*Fig*. 66, *a, and Fig* 67, *a*)

During fermentation most cells are free, medium-sized, and oval

The yeast lies rather loose in the flask, if shaken, it is not distributed equally in the wort, but separates into clots

Film-formation · After 31 to 32 days a distinct film, which, however, does not cover the whole surface, and subsequently develops slowly.

The cells of the film have a very different appearance from those seen in the fermentation-stage Many of them are much lengthened and irregularly twined, some have developed a ramified mycelium.

If developed at a low temperature, the spores are small, coherent, granulated At 11° to 12° C no spores appear within a fortnight, at 25° C., a very scanty spore-formation takes place in forty hours.

When the principal fermentation was interrupted, the liquid contained 2·49 per cent by vol of alcohol, in the following two periods 0·86 and 0·12 per cent. by vol. were formed.

Acid-production 5·2

Agreeable smell and fine aromatic taste

6 (*Fig*. 66, *b, and Fig* 67, *b*)

The cells are round, oval, and elongated during fermentation, all forms occurring in chains, isolated round giant cells occur

The yeast lies rather compact in the flask; it requires strong shaking to distribute the cells equally throughout the liquid.

Film-formation. After 26 days the surface growth forms a ring of yeast cells on the wall of the flask; only slight indications

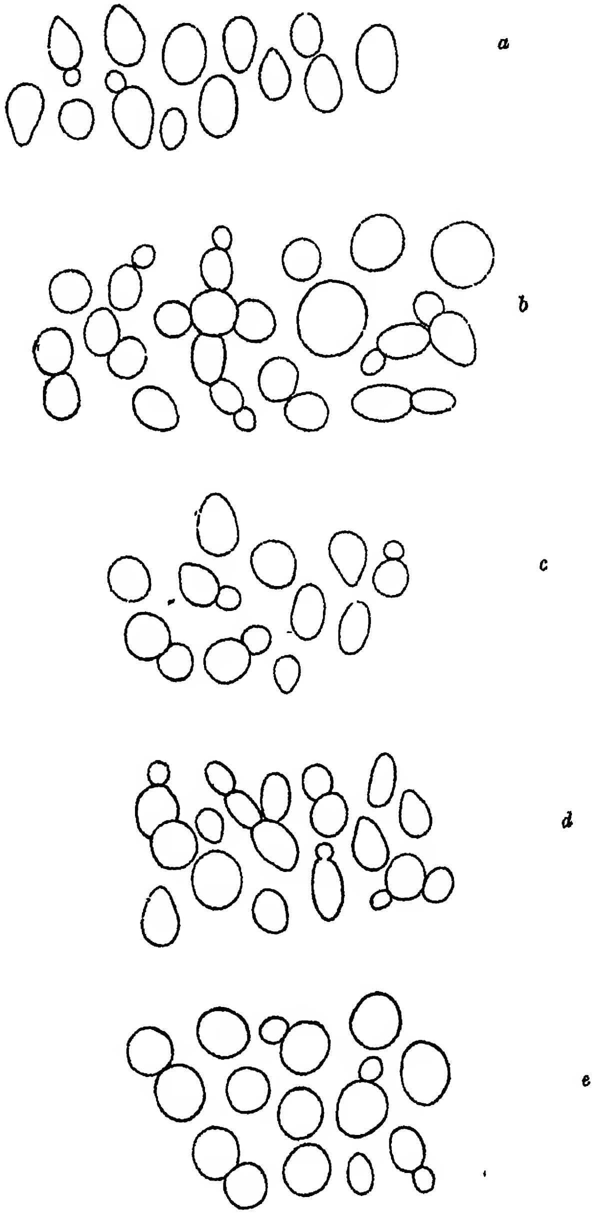


Fig 66 —a-e, Young growths of English top-yeasts (Holm)

of film-formation. After 31 to 32 days the film had not developed further

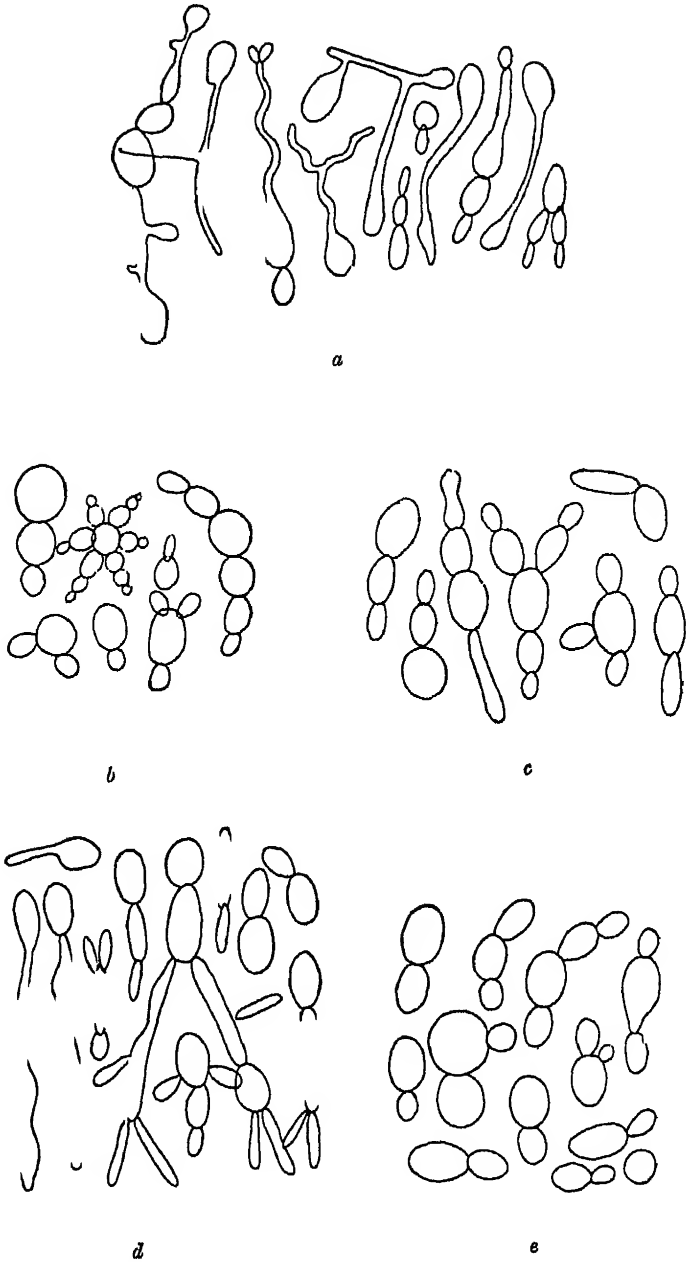


Fig 67.—*a-e*, Film-formations of English top-yeasts (Jorgensen)

The cells of the ring-growth cannot be distinguished from those occurring during alcoholic fermentation

If developed at a low temperature, the spores are comparatively small, granulated, with no distinct vacuoles At 11° to 12° C, for seven days, very few spores are formed, at 25° C., for forty hours, a scanty spore-formation takes place

When the principal fermentation was interrupted, the liquid contained 1 85 per cent by vol of alcohol, during the following two periods 0 65 and 0·2 per cent by vol were formed

Acid-production 6.

Agreeable smell and slightly aromatic taste.

7 (*Fig* 66, *c*, *and Fig* 67, *c*)

During fermentation round and oval cells, some free, others linked in short chains

The yeast lies rather compact in the flask, violent shaking is required to distribute the cells equally throughout the liquid,

Film-formation After 26 days a thin, almost continuous film appears, which in the course of the next five to six days forms a conspicuous covering extending over the whole surface of the liquid.

The cells of the film have in the main the same shape as those seen during fermentation, only the youngest generations are elongated and narrow

If developed at a low temperature, the spores are medium sized, with no distinct vacuoles At 11° to 12° C, after nine days fully developed spores appear, at 25° C, for forty hours, spores are formed freely

When the principal fermentation was interrupted, the liquid contained 2·4 per cent. by vol of alcohol, during the following two periods 0·95 and 0 00 per cent by vol. were formed.

Acid-production 6 5.

Agreeable smell and slightly aromatic taste

8 (*Fig* 66, *d*, *and Fig* 67, *d*)

During fermentation, round, oval, and elongated cells, both free and linked together

The yeast lies rather compact in the flask; on violent shaking the cells are distributed equally throughout the liquid

Film-formation After 31 to 32 days very slight isolated patches of a film on the surface, and a slight ring of yeast-cells on the glass, round the edge of the liquid

The cells of the film have assumed quite different shapes from those of the fermentation-stage, they are very much lengthened, mycelial, and irregular

If developed at a low temperature, the spores are medium-sized, with no distinct vacuoles. At 11° to 12° C. spores are formed pretty freely on the ninth day, at 25° C, they are formed freely in forty hours

When the principal fermentation was interrupted, the liquid contained 2·77 per cent by vol. of alcohol, during the following two periods 0·98 and 0·00 per cent. by vol were formed

Acid-production: 6·5

Odour good, but bitter, persistent taste.

9. (*Fig.* 66, *e*, *and Fig* 67, *e*.)

During fermentation a very uniform growth of big, round and oval cells

The yeast lies rather loose in the flask, on shaking, the cells are distributed equally throughout the liquid

Film-formation. In 31 to 32 days very slight, isolated patches on the surface, and a slender ring of yeast cells on the glass, round the edge of the liquid.

The cells of the film differ but little from those of the fermentation.

If developed at a low temperature, the spores are medium in size and granulated. At 11° to 12° C. spore-formation sets in on the ninth day, at 25° C. for forty hours, a somewhat scanty spore-formation takes place, accompanied by a considerable formation of net-work.

When the principal fermentation was interrupted, the liquid contained 2·96 per cent. by vol of alcohol, during the following two periods 1·19 and 0·00 per cent. by vol. were formed.

Acid-production. 7.

Odour good, pronounced vinous taste

By comparing the figures of these nine races or species the interesting observation will be made that their morphological differences are much more pronounced in the film-vegetations than in those formed during alcoholic fermentation.

Regensburger has since undertaken very detailed comparative experiments with three species of top-fermentation yeasts, which, like the examples just referred to, display characteristic differences in the general appearance of the young growths Spore-formation occurs within differing periods, and conforms to the rule laid down by the author many years ago that the development of spores usually takes place more rapidly with top yeasts than with bottom yeasts. These species also differ very distinctly in the visible course of fermentation, in the cardinal points for skin formation and in the development on solid substrata.

It was at one time assumed that purely-cultivated top yeasts

would quickly become impure on account of the prevailing high temperature Experience has shown that this objection carries no weight, that great progress may be made in this field, and that considerable advantages may accrue from the use of a single selected type A further objection raised was that it is impossible by means of a single species to obtain a stable secondary fermentation, a wrong assumption previously made in connection with low-fermentation yeast Van Laer strongly emphasised this view, and while he freely admitted that low yeast types exist, capable of carrying through a normal secondary fermentation, he believed that the author was wrong in ascribing the same properties to top yeasts. Notwithstanding the practical results attained by exact experiments carried out with selected types, even when due allowance was made for the special English conditions referred to by van Laer, and in spite of the fact that no exact proof was forthcoming to warrant the opposite view, the author's experience was ignored, and van Laer prepared mixtures of top-yeast species which were distributed for use in breweries. They were designed to satisfy practical demands, the intention being that one species should carry through the primary fermentation, the other the secondary fermentation It is true that the possibility is not excluded that such a composite yeast could be prepared, but even when van Laer's preparations gave good results in practice it could not be proved that it was due to the activity of the composite yeast as such It must first be demonstrated that this new yeast really reacted as a composite yeast—*i.e*, that the different constituents are really capable of acting together In conjunction with J C Holm, the author investigated many of the preparations distributed throughout the industry, and it was shown that even during the primary fermentation one of the species very strongly predominated, whilst in the secondary fermentation the other species disappeared Thus the problem of preparing a truly composite yeast had not been solved The experience of subsequent years has always confirmed the correctness of the first results, even in fermentations carried out on the English system. It is possible in both top and bottom fermentations to carry out the whole primary and secondary fermentation with a single selected species

Quite recently the question of using two species in English top-fermentation has been re-opened It has been supposed that the secondary fermentation of stout is brought about by certain species of *Torula* (two are described in the systematic part of this chapter), and that it is due to their activity that this variety of beer acquires its peculiar sourish taste Here, however, two separate facts have been confused The true secondary fermentation is carried out by *the properly selected type* of *Saccharomyces*, and can be regulated like any other secondary fermentation In this

connection the activity of the *Torulas* is unnecessary, as may plainly be seen from the fact that in every European country, and in other parts of the world, as shown by the author, stout and similar kinds of beer can be prepared from one of certain selected types of yeast These species of *Torula* are to be reckoned in the same category as the lactic-acid bacteria, acetic-acid bacteria, Sarcina, etc , which also impart a special taste to a fermented wort. If such a taste is desired, it is of importance to regulate the activity of the organism in question, so that the quantity of the peculiar fermentation or assimilation product may stop short of a given limit If this is exceeded the liquid becomes undrinkable

There seems to be a typical difference between the races predominating in English top yeasts, the North Country yeast (*e g.*, in the Yorkshire stone square system) being characterised as slow fermenting, and that used in Burton and London as fast fermenting.

A most important corroboration of the results arrived at by the writer in regard to the use of single-cell cultures in English top-fermentation is given in Horace T Brown's Reminiscences of the application of scientific methods in brewing practice (1916) This eminent scientist says —" The pure yeast beer, on an average, did not show any marked superiority over those brewed with the ordinary brewery yeast, as regards flavour, brilliancy, and general qualities " This observation really goes to prove that the whole fermentation can be carried through by one single species. Even if the best methodical selection is made from the different races composing brewery yeast, the brewer cannot expect to obtain better results than the normal, providing that the pitching yeast is free from foreign germs and that a good race predominates , but by a rational treatment of the pure-cultivated yeast it becomes possible to guard against all those irregularities of fermentation that may arise from the use of common yeast, which is always a mixture of several races and (as shown by the writer's numerous researches) often contains wild-disease-yeasts. Brown adds that " the pure yeast beers were slower in conditioning than the corresponding samples from the ordinary yeast " But this inconvenience can be remedied by a *methodical* selection from the yeast races and a *correct* treatment of the yeast during the development of the culture and afterwards; for, on the evidence of experiments on many micro-organisms, including yeasts, the maturing required in certain English beers can doubtless be secured and even accelerated by a proper adaptation.

After the author had introduced pure cultures into top-fermentation practice in many European countries, the reform met with general agreement. The work was taken up in its early stages by Kokosinsky, de Bavay, M'Cartie, W. R. Wilson, A Millar,

and R Gray, to give but a few names At a later stage, J Schonfeld introduced selected types of top yeasts into many German breweries

In the case of top-fermentation lactic-acid beers, like German "Weissbier," the rational treatment must consist in the main in first carrying through a lactic-acid fermentation, and then applying the pure-cultivated top yeast

(*b*) Distillery and Pressed Yeasts.

To solve the problem whether distillery and pressed yeasts are capable of forming endogenous spores, a possibility denied by Wiesner and Brefeld, the author, in 1884, undertook an exact examination of a number of samples of such yeasts, and in the same year, together with Hansen, published the results in Dingler's *Polytechnic Journal*, showing that there is no possible difficulty in obtaining an abundant and rapid spore-formation from these species At the same time the author was able to arrive at certain conclusions regarding the composition of such yeast By the help of fractional cultures, it was found that both *top* and *bottom fermentation species* occur in ordinary distillery and pressed yeast Further investigations showed that in one and the same mass of yeast two morphologically different types may frequently occur, one chiefly giving isolated cells in a fully-developed state, the other, budding colonies of many cells It was impossible, however, to trace any connection between this distinction and the fermentation phenomena brought about by the two species The two morphological types remain unaltered after being preserved for years

Detailed researches further showed that yeast used in distilleries includes a multitude of clearly distinguishable types, and a few years later pure selected races from the author's laboratory were first introduced into yeast factories, and then into the distilleries of Northern Europe and into Molasses factories

Owing to the physiological state of the species very important differences were exhibited with respect to propagating power, yield of alcohol, character of the alcohol, etc , and it was necessary in many cases to prepare the absolutely pure culture from the yeast in each individual factory. By expert application of such cultures, and particularly by a rational lactic acidification, which arrests the development of foreign organisms found in the mash, it was possible to secure a higher yield and a better quality both of alcohol and of yeast Rayman and Kruis have undertaken elaborate investigations with regard to the character of the distillate obtained by the use of different species

In 1890 the Berlin Experimental Station sent out the first yeast species cultivated from this group by P. Lindner It was described as *Race 2.* More recently another species, *Race 12,*

has been brought into practical use, and this appears to be preferred according to Lindner's communications, both in potato distilleries and in pressed yeast factories

Henneberg has given a detailed description of both species, from which it may be noted that the giant colonies differ in appearance. In *Race* 2 they have an almost smooth surface, scored by a few shallow, concentric and radial furrows, the outline is fairly straight *Race* 12 has a very uneven surface, scored by deep, irregularly radial furrows The ridges so formed constitute an extremely delicate concentric pattern, and the outline is formed erratically by the termination of the ridges at varying distances from the centre The small colonies in plate-cultures are similar. *Race* 2 has feebly-developed budding colonies, whilst *Race* 12 forms large and dense clusters The cells of 2 are an elongated oval; those of 12 are roundish and oval

(*c*) Wine Yeasts.

When a number of pure growths are isolated from the usual elliptical wine yeasts, it will readily be seen that they vary greatly in morphological character, under similar conditions of cultivation, especially if the general appearance is taken into account Species with both large and small cells and every intermediate form are met with, from elongated and elliptical, to oval and almost spherical. *Pastorianus* forms of yeast also exist Before 1890 a series of such types, exhibiting stable morphological characters, and displaying characteristic differences in spore-formation, had been isolated in the author's institute

Hansen had published further information regarding the individual species in his description of *S ellipsoideus I.* (see following section), and subsequently in his notes regarding *Johannisberg II.* He found that the temperature limits for budding in wort by the last named species are 37° to 38° C and 0 5° C , for spore-formation on gypsum blocks 33° to 34 5° C and 2° to 3° C Further publications we owe to Aderhold, Hotter, Kayser, Marx, Muller-Thurgau, Seifert, Wortmann and others

As examples of the different biological characters observed in wine yeast, we will discuss more closely a few of the species described by Aderhold.

Johannisberg I. has round or oval-pointed cells, in the young film, produced at 26° to 27° C, the cells are oval, spores appear in 28 to 30 hours at 25° to 26·5° C *

Johannisberg II has large oval cells, characterised by longish but blunt ends. The film cells are round, oval, and sausage-shaped, spores are formed within 23 to 24 hours.

Kreuznach has the same cell-formation as the previous species,

* Similar temperatures hold good for the following species.

but somewhat smaller, film cells like *Johannisberg I*, spores in 30 hours

Mulheim has broad, oval, and, less frequently, round cells, with short pointed ends smaller than the previous species; only round and oval film cells, spores within two to three days

Walporzheim I has round cells, the oval forms scarcely pointed; often budding colonies in the film, elongated links forming an axis for the colony surrounded by round cells, spores in 80 hours.

Piesport, predominantly elliptical cells without pointed ends, only spherical cells in the film, spores in 23 to 24 hours.

Grown on solid substrata, differences can be observed in the development of colonies

Extensive researches made by Muller-Thurgau and Wortmann have shown that the wine yeasts may be ranged in characteristic groups Thus, Rhine wine yeasts exhibit a common character by which they distinguish themselves from yeasts native of the Moselle district or of Champagne, and so on These inquirers, therefore, recommend, in general, that the must be fermented with a yeast derived from the region in which the particular wine is grown. By way of example, Muller-Thurgau, in regard to red wine, points out the importance of using only yeast isolated from the corresponding must, as yeast-races from white musts, however excellent they are for these, show a slow growth and weak fermentative power in red musts.

Pure selected races have gradually been introduced in large numbers into wine fermentation by Jaquemin, Kayser, Martinand, Muller-Thurgau, Ritsch, Rommier, Wortmann, the author and others Muller-Thurgau and Wortmann, among others, have indicated and proved the importance of these pure cultures, as the wine is dominated by the pure yeast throughout every stage of its development, extending, it may be, through many years *

In the fermentation of *fruit juices* the use of specially selected yeasts must be of great practical importance, because, as an examination of the juices reveals, tree-fruits in particular generally harbour on their surface far larger numbers of foreign germs capable of development (bacteria and moulds outnumbering the yeasts) than grapes As is well known, various fruit-wines (apple-wine or cider, pear wine or perry, etc) are manufactured in considerable quantities in many countries, and the requisite fermentations have been studied by Kayser, Muller-Thurgau, Wortmann, Barker, Grove and others A few of the species discovered are mentioned in the systematic part of this work The pure cultures employed for such purposes are isolated sometimes from wine yeasts, and sometimes (more reasonably) from various species occurring in spontaneous fermentations of fruit juices, Muller-Thurgau and Osterwalder, using yeasts of the latter class, obtained satisfactory

* See the section on the behaviour of *Saccharomycetes* with sugars, etc

results, more particularly when types were chosen which were not only endowed with high fermenting and inverting powers, but, by developing a suitable amount of non-volatile acids, were also capable of making up for the deficiency of apple- or pear-juice (*Bericht der Schweiz Versuchsst.*, Wadenswil, 1905, 1906; *Landw Jahrb. d Schweiz*, 1908, 1910) Selected wine yeasts appear to be of value in cases where bouquet formation is wanted, as in berry-wines. In the case of *English cider*, Barker and Grove found large quantities of foreign organisms in the raw juice, and pointed out the difficulties thrown in the way of the use of pure cultures by the fact that apple-juice contains only a small amount of albuminoids, so that the yeast is soon weakened and suppressed by bacteria. By pasteurising the must at 70° C, and, when necessary, adding proteids, satisfactory results were obtained (without the product showing a cooked taste), particularly with a yeast isolated from a cider It appears that a biological purification can be effected by means of formaldehyde, which will gradually enter into combination with certain constituents of the juice.

Cocoa Fermentation.—Regarding spontaneous fermentation of cocoa beans, careful biological investigations were made by Davies in Jamaica (*vide* Bibliography, Bainbridge and Davies). The beans with the adhering pulp are placed in large fermenting boxes with false bottoms and loose covers, so that the fermentation proceeds in the presence of traces of air In the course of the first day and night, a temperature of 35° to 40° C is reached, which afterwards rises to 40° to 45° C., and if the fermentation is allowed to proceed further for five or six days, the temperature rises to about 50° C. If the fermentation is continued still further the temperature drops. Every second or third day the mass is completely mixed by transferring to another box During the process, which proceeds vigorously, the surface of the beans changes gradually from white or pale pink to rich brown, while at the same time characteristic odours are developed. Biologically, three stages can be clearly distinguished In the first stage, after some twelve hours, there appear an abundant development of *Sacch. apiculatus* and a comparatively scanty growth of *S. anomalus* Thereafter comes an enormous development of true saccharomycetes with round and oval cells, this growth attaining its maximum on the second day, a quantity of alcoholic liquor at the same time draining away. The third stage is characterised by a rich development of acetic acid bacteria, carried to a large extent into the boxes by the "vinegar fly" (Drosophila). The drainings now consist of dilute vinegar. The true fermentation is then at an end. If the process is continued for eight or nine days more, a development of spore-forming bacteria of the *subtilis* type appears, and the odour which has gradually changed from sweet and fruit-like to strongly alcoholic, ethereal, and then strongly acetic, will finally change to

that of high game The beans are partially freed from pulp during fermentation, and are subsequently dried If this takes place in the sun, a modified fermentation will occur at night, and the surface of the moist beans will be found covered with large colonies of yeast and bacteria In the course of the fermentation just described, the chemical composition of the beans gradually changes A number of the products of fermentation percolate into the bean and the less volatile constituents remain in the dry kernal On subjecting the roasted beans to distillation the early fractions of the essential oil of cocoa were found to be associated with esters derived from the fermentation, which undoubtedly enhanced the quality of the bean A prolonged fermentation results in the loss of some of the carbohydrate and albuminoid constituents of the bean, leaving a substance richer in fat The careful research described has shown fermentation to be of great importance in determining the quality of cocoa.

One of the yeast species concerned was described by Preyer under the name of *Sacch theobromae*, the cells are very short ; each of which is said to form a considerable number of spores at 25° C. in the course of twenty hours It does not invert saccharose, and soon perishes in a solution of this sugar

Further observations regarding cocoa fermentation were made by Preyer (*Der Tropenpflanzer*, 1901), Chalot, and Perrot, Summarised in Hamel Smith's publications

2. Yeasts not yet applied Industrally *

Saccharomyces Pastorianus or Saccharomyces Pastorianus I. Hansen (Figs 68, 69).

Bottom-fermentation yeast

Sedimentary forms developed in wort —Predominantly elongated and sausage-shaped, also large and small oval and round cells (Fig 68) When this species is cultivated in wort near its maximum temperature for growth, its vegetation consists of sausage-shaped and elongated cells The temperature limits for budding in wort are 34° and 0 5° C.

It frequently occurs in the air of fermenting rooms, and is also found in diseased beers It imparts to beer a disagreeable bitter taste and unpleasant odour , it may also produce turbidity, and interfere with the clarification of beer in the fermenting vessel.

According to the investigations of Mach and Portele, this species may also be successfully used in wine fermentation.

* For systematic reasons, however, we have embodied in this chapter several species which are used in some special branches of the industry, such as milk-, rum- and cider-fermentation

Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose It assimilates glucose, maltose, and saccharose in yeast-water, and in solutions of asparagine with nutritive salts

Fig 68 —*Saccharomyces Pastorianus I* (Hansen) —Cell-forms of young sedimentary yeast (after Hansen)

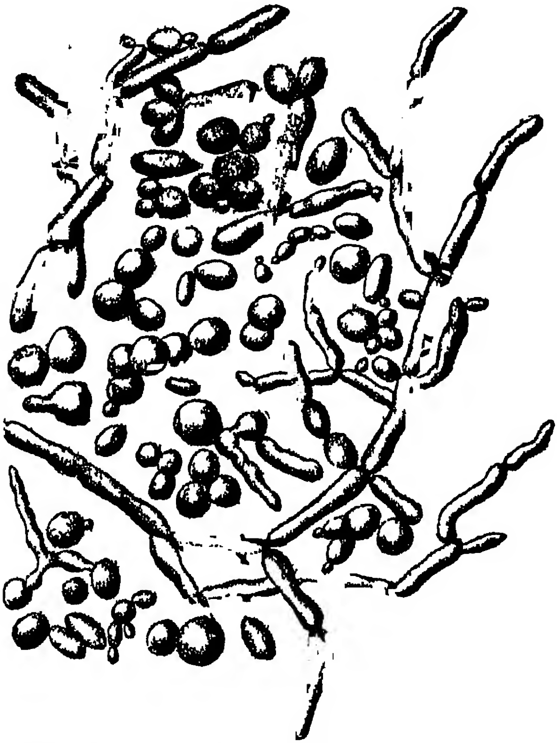


Fig 69 —*Saccharomyces Pastorianus I* (Hansen) —Film-forms at 13° to 15° C., from Holm's drawing in Hansen's Memoir

Ascospore-formation (Fig 58, 2) —

At 31·5° C	no ascospores are developed			
29·5-30·5	the first indications are seen after		30 hours.
29	,,	,,	27 ,,
27·5	,,	,,	24 ,,
23·5	,,	,,	26 ,,
18	,,	,,	35 ,,
15	,,	,,	50 ,,
10	,,	,,	89 ,,
8·5	,,	,,	5 days.
7	,,	,,	7 ,,
3-4	,,	,,	14 ,,
0·5	no ascospores are developed.		

Size of spores 1·5 to 5 μ.

Film-formation :—

At 34° C	no film-formation occurs			
26-28	feebly-developed film-specks are seen after		7-10 days.	
20-22	,,	,,	8-15 ,,	
13-15	,,	,,	15-30 ,,	(Fig 69.
6- 7	,,	,,	1- 2 months.	(Fig 69.
3- 5	,,	,,	5- 6 ,, like Fig 69, but without the large colonies.	
2- 3	no film-formation occurs			

Microscopic appearance of the cells in the films —

At 20° to 28° C almost the same forms occur as in the sedimentary yeast

At 13° to 15° C strongly-developed mycelial colonies of very elongated, sausage-shaped cells are fairly frequent (Fig. 69).

In old cultures of films the cells are smaller than in the sediment, very irregular and sometimes almost thread-like cells are found

Saccharomyces intermedius or Saccharomyces Pastorianus II. Hansen (Figs 70, 71)

Feeble top-fermentation yeast

Sedimentary forms grown in wort —Mainly elongated, sausage-shaped cells, but also large and small, oval, and round cells (Fig. 70).

When this species is cultivated in wort near the maximum temperature for growth its vegetation consists of round and oval

cells. The temperature limits for budding in wort are 40° and 0·5° C

It frequently occurred in Hansen's analyses of air in the brewery, it appears to belong to the species which do not cause diseases in beer Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose.

Ascospore formation (Fig. 58, 3) :—

At 29° C.	no ascospores are developed.			
27-28	the first indications are seen after		34	hours.
25	,,	,,	25	,,
23	,,	,,	27	,,
17	,,	,,	36	,,
15	,,	,,	48	,,
11·5	,,	,,	77	,,
7	,,	,,	7	days.
3-4	,,	,,	17	,,
0·5	no ascospores are developed.			

Size of the spores 2 to 5 μ

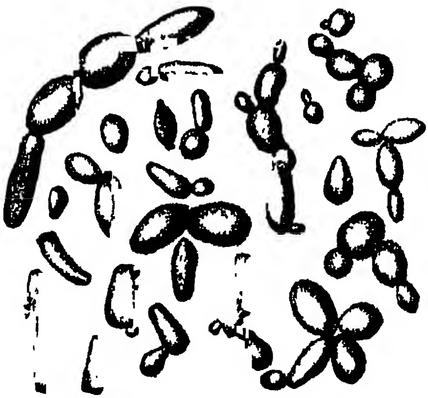


Fig 70 —*Saccharomyces Pastorianus II* (Hansen)—Cell-forms of young sedimentary yeast (after Hansen)

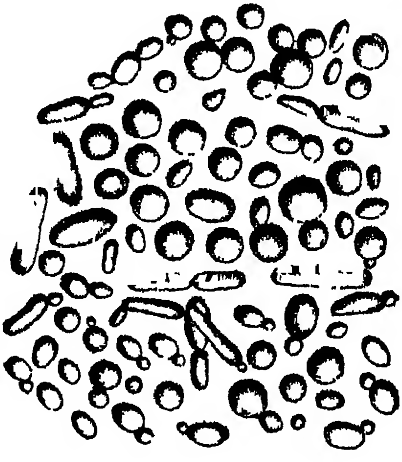


Fig 71 —*Saccharomyces Pastorianus II* (Hansen) —Film-forms at 15° to 3° C (after Holm's drawing in Hansen's Memoir)

Film-formation :—

At 34° C	no film-formation occurs.			
26-28	feebly-developed film-specks are seen after	.	7-10 days.	
20-22	,,	,,	8-15 ,,	
13-15	,,	,,	10-25 ,,	(Fig. 71.)
6- 7	,,	,,	1- 2 months.	(Fig. 71.)
3- 5	,,	,,	5- 6 ,,	(Fig. 71.)
2- 3	no film-formation occurs.			

Microscopic appearance of the cells in the films —

At 20° to 28° C., almost the same forms as in the sedimentary yeast, also irregular sausage-shaped cells.

At 15° to 3° C., mostly oval and round cells.

In old cultures of films the cells are smaller than in the sediment; very irregular and sometimes almost thread-like cells are found.

Streak cultures of this species in yeast-water gelatine give growths with comparatively smooth edges after sixteen days at 15° C., and in this respect it also differs from the following species —

Saccharomyces validus or Saccharomyces Pastorianus III. Hansen. (Figs. 72, 73.)

Top-fermentation yeast.

Sedimentary forms grown in wort —Mostly elongated, sausage-shaped, but also large and small oval and round cells (Fig. 72). When this species is cultivated in wort near the temperature

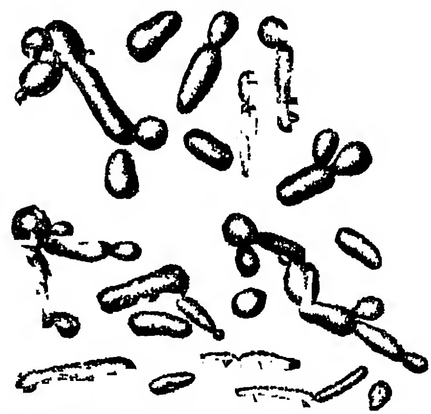


Fig. 72 —*Saccharomyces Pastorianus III.* (Hansen).—Cell-forms of young sedimentary yeast (after Hansen).

maximum for growth, the vegetation consists of round and oval cells. The temperature limits for budding in wort are 39° to 40° C. and 0·5° C.

It was separated from a bottom-fermentation beer which showed yeast-turbidity, and has been proved by Hansen to be one of the species which produce this disease. Recent experiments of Hansen show that this disease-yeast possesses another peculiar property—its addition will in certain cases effect a clarification when the fermenting wort has an opalescent appearance.

According to investigations made by the author, a strong infection of low-fermentation yeast with this species may in certain cases effect an excellent clarification and good "breaking" in

both fermentation vessel and cask Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose

Ascospore-formation (Fig 58, 4) —

At 29° C	no ascospores are developed	
27-28	the first indications are seen after	35 hours.
26·5	" "	30 "
25	" "	28 "
22	" "	29 "
17	" "	44 "
16	" "	53 "
10·5	" "	7 days
8·5	" "	9 "
4	no ascospores are developed.	

Size of the spores 2 to 5 μ

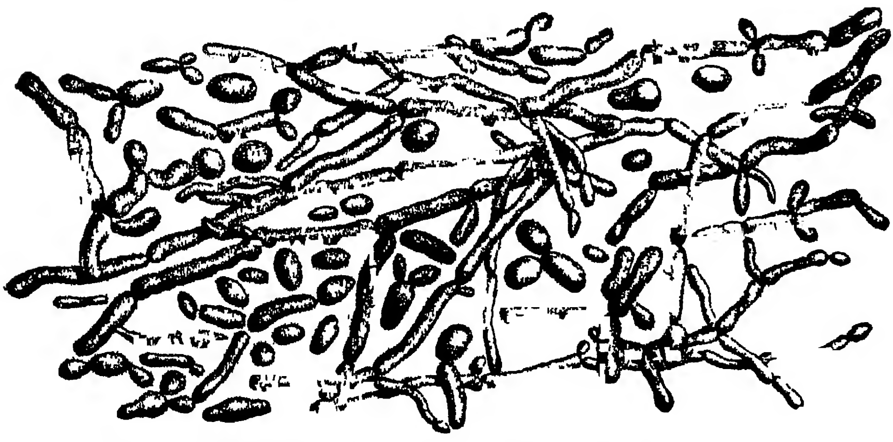


Fig 73 —*Saccharomyces Pastorianus III* (Hansen) —Film-forms at 15° to 3° C, (after Hansen)

Film-formation .—

At 34° C	no film-formation occurs	
26-28	feebly-developed film-specks are seen after .	7-10 days
22-20	" "	9-12 "
13-15	" "	10-20 "
6- 7	" "	1- 2 months (Fig 73)
3- 5	" "	5- 6 "
2- 3	no film-formation occurs	

Microscopic appearance of the cells in the films ·—
At 20° to 28° C · Almost the same forms as in the sedimentary yeast.

At 15° to 3° C Strongly-developed colonies of elongated, sausage-shaped or thread-like cells, which closely resemble a mycelium in appearance (Fig 73)

In old cultures of films, the cells have the same forms as at 15° to 3° C, but are often still thinner and more thread-like

Streak cultures of this species in yeast-water gelatine, after sixteen days at 15° C, give growths with distinctly hairy outline

Saccharomyces ellipsoideus or Saccharomyces ellipsoideus I.
Hansen (Figs 74, 75)

Bottom-fermentation yeast

Sedimentary forms grown in wort —Mostly oval and round cells, sausage-shaped cells are rare (Fig 74).

If this species is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells The temperature limits for budding in wort are 40° to 41° C. and 0·5° C Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose. Assimilates glucose, maltose, and saccharose in yeast-water and in solutions of asparagine and nutritive salts

Occurs on the surface of ripe grapes

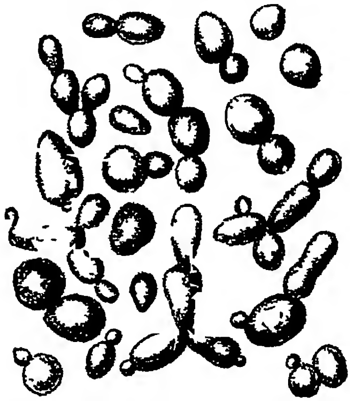


Fig 74 —*Saccharomyces ellipsoideus I* (Hansen) —Cell-forms of young sedimentary yeast (after Hansen).

Ascospore-formation (Fig 58, 5) —

At 32·5° C	no ascospores are developed	
30·5-31·5	the first indications are seen after	36 hours
29 5	,, ,,	23 ,,
25	,, ,,	21 ,,
18	,, ,,	33 ,,
15	,, ,,	45 ,,
10·5	,, ,,	4½ days.
7·5	,, ,,	11 ,,
4	no ascospores are developed.	

Size of the spores 2 to 4 μ.

Film-formation :—

At	38° C	no film-formation occurs			
	33-34	feebly-developed film-specks are seen after	.	8-12 days.	
	26-28	,,	,,	9-16 ,,	
	20-22	,,	,,	10-17 ,,	
	13-15	,,	,,	15-30 ,,	(Fig 75.)
	6- 7	,,	,,	2- 3 months.	
	5	no film-formation occurs.			

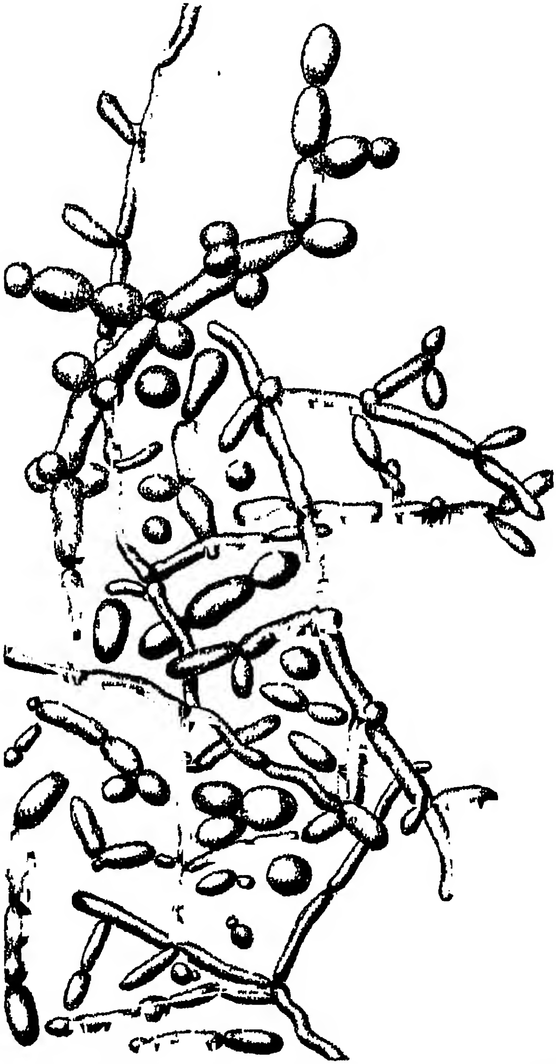


Fig 75.—*Saccharomyces ellipsoideus I* (Hansen) —Film-forms at 13° to 15° C (from Holm's drawing in Hansen's Memoir).

Microscopic appearance of the cells in the films —

At 20° to 34° C and 6° to 7° C the cells are smaller and more sausage-shaped than in the sedimentary yeast.

At 13° to 15° C, freely-branched and strongly-developed colonies of long or short sausage-shaped cells, often with verticillated branches (Fig 75)

In old cultures of films, the cell forms are the same as at 13° to 15° C

Streak cultures of this species in wort-gelatine (wort with the addition of about 5·5 per cent. of gelatine), in the course of eleven to fourteen days at 25° C, give—in contradistinction to the other five species—a characteristic net-like structure, by means of which it can be distinguished by the naked eye from other species

Saccharomyces turbindans or Saccharomyces ellipsoideus II. Hansen (Figs 76, 77)

Usually a bottom-fermentation yeast

Sedimentary forms grown in wort —Oval and round cells predominate, sausage-shaped cells are rare (Fig 76)

Fig 76 —*Saccharomyces ellipsoideus II* (Hansen) —Cell-forms of young sedimentary yeast (after Hansen)

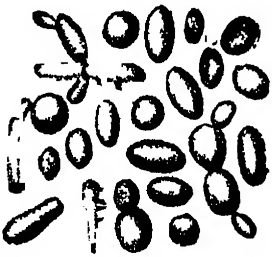


Fig 77 —*Saccharomyces ellipsoideus II.* (Hansen) —Film-forms at 28° to 3° (after Hansen)

It was separated from beers which showed yeast-turbidity; is a species which causes yeast-turbidity, and is more dangerous than *Sacch Pastorianus III* If this species is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells The temperature limits for budding in wort are 40° C and 0·5° C Saccharose, dextrose, lævulose, and maltose are fermented, but not lactose Assimilates glucose, maltose and saccharose in yeast-water

Ascospore-formation (Fig 58, 6) —

At	35° C	no ascospores are developed		
	33-34	the first indications are seen after	31	hours
	33	,, ,,	27	,,
	31·5	,, ,,	23	,,
	29	,, ,,	22	,,
	25	,, ,,	27	,,
	18	,, ,,	42	,,
	11	,, ,,	5½	days.
	8	,, ,,	9	,,
	4	no ascospores are developed		

Size of spores 2 to 5 μ.

Film-formation —

At	40° C	no film-formation occurs		
	36-38	feebly-developed film-specks are seen after	8-12 days	
	33-34	,, ,,	3- 4 ,,	
	26-28	,, ,,	4- 5 ,,	(Fig 77.)
	20-22	,, ,,	4- 6 ,,	(Fig 77.)
	13-15	,, ,,	8-10 ,,	(Fig 77.)
	6- 7	,, ,,	1- 2 months	(Fig 77.)
	3- 5	,, ,,	5- 6 ,,	(Fig 77.)
	2- 3	no film-formation occurs		

Microscopic appearance of the cells in the films ·—

At all temperatures, the same forms as in the sediment, at and below 15° C, the cells are only slightly more elongated (Fig. 77).

A particular species causing turbidity in beer and, when pure cultivated, a disagreeable pungent taste, was studied by Christoph. Its specific characteristic is the copulation of two spores before germination At 25° C its multiplying power is five times less than that of culture yeast Despite its inferior power of resistance it is apt to produce turbidity in beer undergoing the secondary fermentation, and also to impede filtration It ferments maltose, glucose, saccharose, and lævulose

Saccharomyces Willianus Saccardo

is a disease yeast described by Will as having elliptical cells In the old films strongly branched budding-colonies occur consisting of very long cells The maximum temperature for spore-formation is 39° C, at the optimum temperature (34° C) the first traces of spores occur in eleven hours The lowest limit for spore-formation is 4° to 5° C The limits for film-formation are 41° and

4° C The vegetative cells are killed when heated in sterilised wort for half-an-hour at 70° C It forms colonies in wort gelatine, which in the young state (both embedded and on the surface) form a network of wide mesh They afterwards become denser in the middle with irregularly fringed edges Sometimes, however, under the same conditions, compact colonies with regular outline are formed

This species imparts a peculiar sweet taste to beer, followed by a rough bitter after-taste, even in presence of very minute quantities (0 1 per cent) in the pitching yeast The beer is often rendered turbid by this wild yeast after two months at 4° to 5° C. This yeast has a strong fermenting and propagating power, and is very dangerous for beer

Saccharomyces Bayanus Saccardo

was discovered by Will in turbid beer The cells are elliptical In old films strongly branching budding-colonies occur The temperature limits for spore-formation are 32° and 0 5° C , the optimum is 24° C The limit for existence of the vegetative cells in wort is 70° C

Besides causing yeast turbidity this species also imparts a sweetish, disagreeable, aromatic taste to beer, and an unusually bitter and astringent after-taste The odour is aromatic like that of rotten fruit With mixtures of about 29 per cent. of the wild yeast the flavour is very strongly pronounced, and may be recognised even in the presence of 5 per cent. The yeast causes a discoloration of beer. It turns paler, and assumes a foxy appearance

Saccharomyces Logos Van Laer

was derived from a yeast in Logos & Co 's brewery in Rio de Janeiro, and is a bottom-fermentation yeast of a *Pastorianus* shape, which occurs in the fermenting vessel as a loosely-lying sedimentary yeast of cheesy appearance, consequently the beer clarifies very rapidly The fermentation is carried out at high temperatures (22° to 30° C). The yeast ferments very slowly, but gradually produces very high percentages of alcohol The flavour of the beer is entirely different from that of ordinary lager beer According to Rothenbach this species is able to ferment about half of a diastase-dextrin prepared according to Lintner's recipe, but is distinguished from *Schizo-saccharomyces Pombe* by fermenting other kinds of dextrin According to Prior and Weigmann, it ferments Achroodextrin III (Prior) completely, whilst Achroodextrin II is only fermented to the extent of 75 per cent

Meissner determined the action of different acids on the Logos

yeast (in comparison with Saaz and Frohberg) using a nutritive liquid consisting of 10 per cent cane-sugar solution with 10 per cent of yeast-water The fermentative activity of Logos yeast was reduced on the addition of 0·375 per cent of acetic acid (the other two yeasts with 0 25 per cent) A smaller quantity of acetic acid (0·125 per cent) caused the Logos yeast to bring about inversion and to begin fermentation more rapidly It can also withstand larger quantities of lactic acid than the other two species In the presence of lactic acid the formation of alcohol is reduced, but by the addition of 0·125 per cent the Logos yeast gives more acid than usual In general the production of acid is considerably greater in the presence of lactic than in the presence of acetic acid In the presence of both acetic and lactic acids the formation of volatile acids is considerably greater than that of non-volatile The fermentative energy (four days) is greater in presence of acetic acid than in the absence of acid, but the addition of lactic acid considerably restricts it

According to Korff, non-volatile acids are formed in larger quantities in aerated cultures, on the other hand, more volatile acids are formed when hydrogen is passed through the solution

According to Bau, Logos yeast does not contain melibiase, whereas Lindner arrived at the opposite result Further research has shown that both authors are right, for a similar subdivision of the race takes place as in the case of *Torula colliculosa* (see below)

Saccharomyces thermantitonum Johnson

is a yeast found on eucalyptus leaves, and was accidentally grown in a flask which had been infected at a temperature of 84° C The cells had not, however, been killed at this high temperature. By subsequent repeated observations, it was found that this species reacts best at high temperatures Johnson used a temperature of 50° C. as the pitching temperature for fermentations on the large scale The wort need not, therefore, be cooled so strongly as usual, and the yeast can be applied in tropical countries where ice machines are not available Within seventeen hours it is said to furnish a properly and completely fermented beer It does not grow at temperatures under 10° C, and its optimum temperature for propagation and fermentation lies between 40° and 44° C. The cells are small and oval They agglutinate and form clots, which sink rapidly and adhere closely to the bottom Consequently, the liquid clarifies very rapidly

A culture which had been preserved for many years in the writer's laboratory was examined by Euler (*Biochem. Zeits* 97, 1919), who made the interesting observation that the limits of temperature characterising the original culture of 1905 had undergone considerable alteration, the yeast now grew most freely

from 35° to 40° C, and its fermentative power gradually declined at 40° C This result may be accounted for by the assumption that the yeast has been successively adapted to lower temperatures

Saccharomyces Ilicis Gronlund

which was found on the fruit of *Ilex Aquifolium*, is a bottom-fermentation yeast, consisting mainly of spherical cells The temperature limits of spore-formation are 38° and 8° C The spores have no vacuoles In the films slightly-elongated cells are found Streak cultures on gelatine have a floury, but otherwise variable, appearance This species, grown in wort, imparts a disagreeable, bitter taste According to Schjerning, it contains invertase, and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose In ordinary beer-wort it can yield about 2 8 per cent of alcohol (by volume)

Saccharomyces Aquifolii Gronlund

was also found on the fruit of *Ilex Aquifolium* It is a top-fermentation yeast, and consists of large round cells The temperature limits for spore-formation are 31° and 8° C, the spores contain vacuoles In the films, spherical and egg-shaped cells alone occur Streak cultures in gelatine vary in appearance, some being glossy and some floury This species imparts to wort a disagreeable, sweet taste, with a bitter after-taste It inverts saccharose and induces alcoholic fermentation in solutions of saccharose, dextrose, and maltose. In ordinary beer-wort it can yield about 3 7 per cent. of alcohol (by volume)

Saccharomyces pyriformis Marshall Ward

(see Ginger-beer Plant)

Saccharomyces Vordermanni Went and Prinsen-Geerligs

was discovered in Java as an essential factor in the manufacture of arrack It is distinguished by its powerful action as an alcoholic ferment and yields a fine distillate, on account of which it is made use of, in pure culture, in the manufacture of arrack The cells are ellipsoidal, and form not more than four spores This species ferments dextrose, maltose, and saccharose, and contains invertase.

Saccharomyces Saké Yabe.

Y Kozai has published the following conclusions regarding this yeast fungus He discovered it in Koji, and has utilised it with success in a pure condition for the preparation of Saké The cells are chiefly round, and form no large budding colonies. In

older cultures giant cells occur Indications of spores occur within 36 hours at 40° to 41° C (maximum temperature), in 14 hours at 30° to 32° C (optimum temperature), and in 15 days at 3° to 4° C (minimum temperature) The spores (usually from one to three in each cell) are strongly refractive It ferments with ease saccharose, maltose, *d*-mannose, *d*-fructose, glucose, and methyl-glucoside, and with greater difficulty trehalose and *d*-galactose, but not rhamnose nor lactose It splits up melitriose into melibiose and fructose, but it cannot hydrolyse melibiose K Yabe's investigations proved that rice straw is the source of the yeast, a straw used for the preparation of mats which serve to cover up the Koji

Saccharomyces Batatæ Saito.

K Saito has described a Saccharomyces which is of importance in the preparation of a yam brandy, as it is prepared on one of the Japanese islands The species is found in fermenting mash (moromi) prepared from Koji and steamed yams, and is the most active organism in promoting alcoholic fermentation The cells are oval and elliptical, and *Pastorianus* forms often occur in the film Indications of spores occur in twenty hours at 25° C The spores are round, strongly refractive, and usually occur two to three in each cell In ordinary beer-wort 3 per cent by volume of alcohol was produced in ten days at 25° C

Dextrose, lævulose, saccharose and maltose are easily fermented, galactose and raffinose with difficulty, melibiose, lactose, inulin, and *d*-methylglucoside are not fermented

Saccharomyces anamensis

(*Levure anamite*)

from sugar-cane and mash (Cochinchina) was selected for use in the well-known Amylo-process on account of its high fermentation temperature, 35° to 38° C It was described by Heinrich and Will. It is a top-fermentation yeast with oval and round cells, partially elongated in the film, developing round spores at 25° C in 20 hours, their optimum being 33° C (9 hours), maximum 35° C, minimum 12° C The upper limit of growth is between 39 5° and 43° C, according to the nature of the medium The cells are killed at 54° to 56° C Single-cell cultures in gelatine have a regular nucleus with budding agglomerations jutting out from the edge It ferments and assimilates glucose, lævulose, galactose, saccharose, raffinose and maltose, lactose is assimilated, but weakly fermented In maize-mash it produces a particularly active fermentation In mineral media (0 025 per cent $MgSO_4$ + 0·5 per cent KH_2PO_4 + 0·5 per cent asparagine in tap-water) the sugars most readily assimilated are raffinose and maltose

Saccharomyces cartilaginosus Lindner

was discovered by Matthes in Kephir The cells are oval or elongated, with curious granular protoplasm A true film-formation does not take place On the other hand, at the completion of the fermentation, clearly isolated islands of somewhat dense and almost cartilaginous consistency appear on the surface of the wort It ferments saccharose and maltose, but not lactose

Saccharomyces multisporus n.sp.

is an elliptical wild yeast which was found in a few cases in English top-fermentation yeasts by J C Holm Many very large round cells (giant cells) are found amongst the elliptical, within which nine to eleven spores may form, whilst the elliptical cells occur with two to four spores The spores are round and strongly refractive At 25° C ripe spores are formed in forty hours, at 15° C. in seventy-two hours The yeast is a bottom-fermentation species, and adheres so closely to the flasks that it can hardly be loosened by shaking It forms as a thin film, which covers the sides of the flask. In ordinary wort it yields about 4 per cent by weight of alcohol The taste is strongly bitter It ferments dextrose, maltose, and saccharose By preservation both in saccharose and in wort, the giant cells lose their power to produce such an exceptionally large number of spores, in gypsum-cultures most of these cells did not develop spores, but many vacuoles

Saccharomyces mali Risler Kayser

is found in cider. The cells are round, and it does not form a film. At 15° C. spores appear in 96 hours It is a bottom yeast, which ferments saccharose, dextrose, and maltose The sedimentary yeast adheres closely to the flask

Rose isolated from an oak *mucilage* a bottom-fermentation yeast, which is of peculiar interest on account of the facility with which it forms spores In hanging drops of wort almost all cells, after the growth has ceased, form spores in a very short time, generally four in each cell It ferments saccharose, maltose, glucose, and fructose

Saccharomyces mandshuricus

was found by Saito as the predominating fungus in the so-called Chiidzu (Chinese yeast), which is added to a mash of steamed sorghum, that is distilled off after fermentation (Manchurian spirits). Chiidzu is made from barley and green Adzuki beans,

which are crushed and mixed with water to form a compact dough, which is moulded to tile-shaped cakes, these are placed in moist rooms and develop luxuriant growths on their surface and in their interior. After being ground, they are added to the mash. The species has spherical and oval cells 6 to 8 μ large, the spores are globular and generally four to a cell, they develop at 26° to 27° C in 19 to 21 hours Slow film-formation on wort Optimum for growth and fermentation 30° to 35° C Temperature limits for budding 39·5° to 40·5° C and 0·5° to 5° C It ferments glucose, lævulose, mannose, galactose, maltose, saccharose, and raffinose. An asporogenous variety occurs in giant-colonies at low temperature, which, unlike the spore-forming, liquefies gelatine

Saccharomyces Tokyo and Yeddo

were found by Nakazawa in Japan Saké They are peculiar in their manner of forming films on unhopped wort + 3 per cent glucose, which are limited to dry islets, the central point of which, in S Tokyo, is in high-relief, but deeply depressed in S Yeddo The islets scarcely merge into each other, in S Yeddo, however, after a long time, a continuous slimy film is formed in which the islets remain fairly distinct These species are killed at about 60° C They ferment saccharose, maltose, glucose, and galactose

Saccharomyces Marxianus Hansen.

This species, which was discovered by Marx on grapes, and described by Hansen, develops in beer-wort in the form of small oval cells, similar to those of *Sacch exiguus* and *ellipsoideus* Elongated, sausage-shaped cells, often in colonies, soon appear, however, and if the culture is allowed to stand for some time, small mould-like particles are formed, some of these swim in the liquid, others settle to the bottom These particles consist of mycelial colonies of practically the same character as the film-formations of Hansen's six species, they are also built up of cells, which are readily separated at the point of union When *S Marxianus* is cultivated in wort near the maximum temperature for growth, the vegetation consists of round and oval cells The ascospores are kidney-shaped, spherical, or oval After cultivation for two to three months in wort contained in two-necked flasks, there were only traces of film-formation with few sausage-shaped and oval cells

This yeast is one of those species which develop a mycelium under certain conditions of culture on a yeast-water gelatine

In beer-wort it yields only 1 to 1·3 per cent by volume of alcohol, even after long standing. It does not ferment maltose, it inverts saccharose, and in nutritive solutions of the latter,

and of dextrose, it yields considerable quantities of alcohol. It assimilates glucose, maltose, and saccharose in solution of asparagine with nutritive salts, and in peptone-salt solutions; also lactose (Klocker).

The temperature limits for budding in wort are 46° to 47° C. and 0 5° C The maximum temperature for spore-formation lies between 32° and 34° C, the minimum between 4° and 8° C., the optimum between 22° and 25° C. The growth yields quicker and more abundant spore-formation if cultivated in yeast-water, or wort with 10 per cent dextrose

In agreement with his theory that maltose is split up by a particular enzyme differing from invertase, and only subsequently fermented, Emil Fischer found that an aqueous extract of the pulverised growth of this yeast decomposed saccharose, but not maltose

Saccharomyces Coreanus

was isolated by Saito from a Corean sample of "Chinese yeast" It has spherical, oval, and elongated cells, which form spherical spores at optimum 33° to 34° C Ferments saccharose, glucose, fructose, galactose, melibiose, and raffinose From a solution of glucose in yeast-water 6·2 per cent of alcohol was produced There occurred a variety with considerably larger cells and spores

Saccharomyces exiguus (Reess) Hansen

develops a growth in wort, the cell-forms of which most closely correspond to the species described by Reess under the above name.

This species only gives scanty spore-formation and weak film-formation, but it yields a well-developed yeast ring The cells of the film resemble those of the sedimentary yeast, but short sausage-shaped and small cells are more frequent

Hansen found this species in pressed yeast It develops a great fermentative activity in solutions of saccharose and dextrose In wort only small quantities of alcohol are formed It does not ferment maltose solutions It inverts saccharose

Experiments, carried out by Hansen in practice, have shown that this species does not produce any disease in beer, even when present in considerable quantities either at the beginning or end of the primary fermentation, or when it is added after storage.

This is of special interest, as *Sacch exiguus* was formerly regarded as a disease-producing species

Saccharomyces Lindneri

isolated by Guilliermond from an alcoholic ginger drink (West Africa), has ellipsoidal cells, in old cultures irregular forms and

giant cells Limits of budding 5° and 41° C Forms a yeast ri but no film The spores are round and often show copulatio Sets up fermentation in wort, but ferments only saccharos lævulose, *d*-mannose, and (feebly) glucose

Saccharomyces Chevalieri

isolated by Guilliermond from wine from West Africa, h round, oval, and elongated cells Limits for budding 5° and 40° 41° C Spores round, formed at 25° to 30° C in 12 hours Germin tion as in Johannisberg II, but often without copulation Is top-yeast, producing fermentation in wort Ferments saccharo. glucose, lævulose, and *d*-mannose

Saccharomyces Mangini

isolated by Guilliermond from a vinous drink from the tubers Osbeckia Ellipsoidal cells forming yeast ring, but no film Spor develop as in the preceding species Ferments saccharose, gluco lævulose, and *d*-mannose, further, lactose, galactose, and dextr (Guilliermond, *The Yeasts*, 1920)

Saccharomyces Jorgensenii Lasché.

The growth consists of small round and oval cells The optimu temperature for spore-formation is 25° C, the temperature limi being 8° and 30° C. At temperatures above 30° C the grow dies rapidly A true film-formation has not been observed, old cultures only a very feeble yeast ring forms, consisting round and oval cells In gelatine it yields colonies which resemb those of low-fermentation brewery yeast Wort-gelatine is slow liquefied The streak-culture is of a dirty grey colour, and h. smooth edges. This species ferments saccharose and dextrose, b not maltose Consequently it is suppressed when mixed wi cultivated yeasts and grown in malt-wort In wort it yields on 0 89 per cent by weight of alcohol In "temperance beer according to Lasché's statement, it produced a strong turbidity

Saccharomyces lactis γ

isolated by Dombrowski from a dry culture of lactic bacteria, h round and oval cells, with large vacuoles and rich in fat Spor formed on gypsum blocks at 25° C in 76 to 96 hours contain an o drop It is characteristic of this species that the cells in spor culture put forth beak-shaped protuberances Produces a we. top-fermentation and ferments saccharose, glucose, and galactos but not lactose nor maltose.

Saccharomyces Zopfii Artari

was discovered by Zopf in the syrup of a sugar factory. The cells are small, spherical, or elliptical. The temperature maximum for budding in wort is 33° to 34° C, the optimum 28° to 29° C. The temperature maximum for spore-formation is 32° C, optimum 26° to 29° C The spores (usually two in each cell) are round. They are easily produced both in liquid and on solid substrata, but especially well in tartaric acid solution containing potassium nitrate The fungus can supply its demand for carbon from saccharose, glucose, and mannite, but not from maltose, lactose, galactose and inulin It is capable of fermenting a saccharose solution containing 50 to 60 per cent of sugar During fermentation an acid is produced which at a later stage is used up.

According to Artari, its nitrogen requirements can be satisfied by ammonium sulphate When the species is cultivated in a dextrose solution containing from 5 to 8 per cent. of ammonium sulphate, transverse walls make their appearance between the mother and daughter cells, as in the case of *S Ludwigii*. In such a solution only spherical cells occur The addition of potassium nitrate brings about an alteration to pear-shaped cells.

This species is endowed with an exceedingly high power of resistance to high temperatures. In the dry it was found by Artari to stand five minutes warming up to 130° C. It is dangerous in sugar manufacture, because it will withstand a moist heat of 67° C According to recent researches by Owen, it occurs commonly in North American sugar-house molasses, and its thermal dead-point in that product lies between 90° and 100° C Heating to 78° C in the cans of syrup proved insufficient to prevent the yeast-cells from inducing fermentation in the cans; accurate experiments showed that a pure growth heated to 90° C. for ten minutes was still capable of setting up fermentation in concentrated syrup (50 to 60 per cent)

Saccharomyces Bailii Lindner

was isolated from Jopen beer-wort which had a primary concentration of 53° to 54° Balling The cells are large, thick-walled, and elongated, and assume irregular shapes in old cultures (like *Amœba*) The spores are strongly refractive There is no film-formation It ferments dextrose and saccharose, but not *d*-galactose and *d*-mannose It gives a feeble fermentation in wort, and in old cultures the wort has a slightly perfumed odour. It forms the main constituent of yeast in Jopen beer samples, and doubtless plays a part in the preparation of Danzig Jopen beer.

Saccharomyces Rouxi Boutroux

was isolated from fermenting fruit juices, and appears to have been found by Roux in dextrose It has a remarkable action on the sugars It ferments dextrose and maltose, but does not ferment saccharose and lactose The cells are small, round, or oval, and linked together in chains One, two, and three spores occur in the cells, and are also found in the film cells The film does not cover the whole surface of the liquid, but forms islands of yeast dotted over the surface

Saccharomyces Soya Saito

found in Japanese Soja (Shoju), which contains 15 to 17 per cent. of sodium chloride Saito found two different *Saccharomycetes*, which, he affirms, have an aromatic effect on the Soja fermentation. *S. Soya* occurs in larger quantities than the second species The cells are round or oval The spores are formed in the yeast ring, and preferably in abnormally constructed cells Saito did not succeed, however, in obtaining spores on gypsum blocks after the yeast had been cultivated in wort or in dextrose yeast-water.

It ferments glucose, maltose, lævulose, *d*-galactose, and *d*-mannose, but not saccharose, lactose, melibiose, raffinose, inulin, or α-methylglucoside According to Saito, it contains invertase in the form of an endoenzyme

Associated with this species a yeast was found forming spores in the film The cells are round or oval, and occasionally mycelial forms are noted It forms a white floury film on koji decoction. Abundant quantities of carbon dioxide collect under the film, and the latter soon assumes a yellowish-brown colour, and displays grooved wrinkles In beer-wort it gives a top-fermentation, but no film-formation The spores are round

It ferments glucose, maltose, and lævulose, but not lactose, galactose, saccharose, melibiose, raffinose, inulin, or α-methylglucoside

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Saccharomyces mali Duclaux Kayser

was discovered in cider, to which it imparts a good bouquet and body The cells are oval, and film-formation takes place At 15° C spores are formed in 84 hours It is a top-fermentation yeast, and ferments dextrose, but not saccharose, maltose, and lactose The sedimentary yeast lies very loosely in the flask

Saccharomyces unisporus n.sp.

was discovered by J C Holm in Dutch whey As regards its behaviour to the sugars, it is most nearly allied to *S mali Duclaux*,

for it ferments dextrose, but not saccharose, maltose, and lactose The cells are small and oval *Pastorianus* forms are also found in old cultures The spores are round and refractive. Only one fairly large spore is found in each cell At 25° C a few cells are found with ripe spores in 40 hours, and at 15° C. in 72 hours No true film-formation occurs, but, on the other hand, a yeast ring is formed in old cultures

Saccharomyces flava lactis Krueger.

A yeast cultivated from cheesy butter must be alluded to, which imparts a curious yellow colour, forms yellow colonies on gelatine, and a yellow film on milk The cells are small, elliptical, and linked in chains It ferments dextrose with difficulty, and lactose not at all. On slices of carrot it quickly forms spores. The most favourable temperature for its growth lies between 18° and 20° C It grows better on slightly alkaline or neutral substrata than on acid It quickly liquefies gelatine It only produces colouring matter when it is in contact with air

Levure de sel *a*

was discovered by Kr Hoye in air analyses which were carried out along the coast of Norway (Bergen and Christiansund). He used wheat paste mixed with about 17 per cent of sodium chloride as the substratum The yeast is round, and forms only a single spore in each cell It thrives best in a fish broth containing 10 per cent. of sodium chloride In a nutritive liquor which contains less than 3 per cent of salt the growth ceases The cells do not alter their round shape in nutritive liquids with varying quantities of salt. It produces no fermentation in apple juice

Saccharomyces Hansenii Zopf

was discovered amongst the fungi of cotton-seed meal It forms spherical spores of very minute diameter, which are developed singly or never with more than two in a mother cell In fermentable saccharine solutions it produces no alcoholic fermentation, but crystals of calcium oxalate are observed in the sediment. Zopf found a similar formation in nutritive solutions containing galactose, grape-sugar, saccharose, lactose, maltose, dulcite, glycerine, and mannite.

Saccharomyces minor Engel.

The vegetative cells are completely spherical, measuring about 6 μ in diameter, and are linked in chains or specks containing six to nine cells The spore-forming cells measure 7 to 8 μ, and contain from two to four spores of 3 μ diameter

Engel believes this organism to be the most active ferment in the fermentation of bread The author has frequently found this minute spore-forming yeast in the sour dough of the Copenhagen bakeries

Saccharomyces hyalosporus Lindner

forms a thin film on wort, and produces no fermentation in the different kinds of sugar The spores are round, and resemble glass beads carrying a lustrous granule in the centre It forms spores in the film The cells are oval, and sometimes rather elongated They are often linked together in chains

It was discovered by Lindner in a sample of beer from a propagating apparatus

Pichia membranæfaciens or Saccharomyces membranæfaciens Hansen.

This peculiar species, which occupies a special place amongst the Saccharomycetes, yields a strongly-developed light grey wrinkled film when grown in wort, which rapidly covers the whole surface of the liquid, and consists mainly of sausage-shaped and elongated oval cells, these have strongly-developed vacuoles, and a more or less empty appearance Separating the colonies is an abundant admixture of air

The limits of temperature for budding on wort are 35° to 36° C, and 0·5° C When this species is cultivated near the limiting temperature, it occurs entirely as sedimentary yeast

According to Seifert, it grows even in presence of 12·2 per cent by volume of alcohol

The spores are very abundantly developed, not only under the ordinary conditions of cultivation, but also in films. They are irregular in form, and at the ordinary room-temperature, germinate in a Ranvier chamber in ten to nineteen hours

On wort-gelatine, the cells form dull grey specks, often with a faint, reddish tinge, which are rounded, flat, wide-spread, and wrinkled The colonies embedded in the gelatine present, however, a very different appearance The gelatine is liquefied by this fungus

This species is incapable of fermenting either saccharose, dextrose, maltose, or lactose, neither does it invert saccharose

According to more recent researches by Klocker, however, it forms minute quantities of alcohol in glucose and lævulose solutions The alcohol formed is quickly converted into acetic acid, and finally into carbon dioxide and water

It was found in the slimy secretion on the roots of the elm, and shows considerable resemblance to the species *Mycoderma cerevisiæ* and *Mycoderma vini*

The maximum temperature for spore-formation is 33° to 33.5° C., the minimum temperature 3° to 6° C., the optimum lying near 30° C. (17 to 18 hours).

It is impossible to prepare an asporogenous variety of *S. membranæfaciens* by cultivation at any temperature lying between the maximum for spore-formation and the maximum for budding.

Koehler found this species in highly polluted well water.

In the writer's laboratory the species was detected in bright wines.

Related to this species is *Pichia Mandshurica*, detected by Saito in "Chinese yeast," used in making Manchurian spirits. In giant colonies on wort-agar it develops dark reddish and yellowish sectors, the former containing spore forming, the latter asporogenous cells. Dextrose is feebly fermented.

Pichi has described two species, one of which, *Pichia membranæfaciens II.*, or *S. membranæfaciens II.*, is found on the leaves of *Euonymus europæus*; the other, *Pichia membranæfaciens III.*, or *S. membranæfaciens III.*, was prepared in a pure state from a wine (Vin des Côtes). Seifert, again, has described three species, *Pichia californica* or *S. membranæfaciens*, var. *californicus*, from a Californian red wine; *Pichia taurica* or *S. membranæfaciens*, var. *tauricus*, from Crimean wine; and *Pichia Tamarindorum* or *S. membranæfaciens*, var. *Tamarindorum*, which was observed on tamarind pulp, and thence found its way into the vinous drink prepared from it.

Klocker describes four new forms: *P. suaveolens*, with spherical or slightly flattened spores; ferments saccharose and glucose, and gives a smell of fruit-ether. *P. alcoholophila*, with spherical or hemispherical spores; ferments dextrose. *P. polymorpha*, with spherical spores and very variable cell forms; ferments saccharose, glucose, and small quantities of maltose. *P. calliphoræ*, with round, angular, or semi-globular spores; ferments dextrose. The first three species were found in soil, the fourth in a fly.

Pichia farinosa or *S. farinosus* was discovered by Lindner in Danzig Jopen beer (53° to 54° Balling), and by K. Saito in Soja sauce. The cells are slim, the older ones often angular. In the film abundant spore-formation takes place. The maximum temperature for the formation of the film is approximately 37° C. This species liquefies wort-gelatine when allowed to stand for some time. It feebly ferments dextrose and lævulose, but not *d*-mannose.

Pichia Radaisii or *S. Radaisii* is described by Lutz, and is found in "Tibi." It is an exciter of fermentation contained in the fig cactus (*Opuntia*), which is used in Mexico in the preparation of a feebly acid and alcoholic drink. The cells are longish oval, the spores round, and usually four in each cell. At 22° to 23° C.

spores form in twelve hours The maximum temperature for spore-formation lies between 25° and 28° C , the optimum temperature for film-formation 23° C Development ceases at 37° to 38° C Colonies on gelatine gradually acquire a red colour

Guilliermond further isolated from Mexican Pulque an allied species, which shows a typical film-formation with round and oval cells, and an abundant formation of hemispherical spores (one to four in each cell) Optimum temperature of spore-formation 29° to 32° C , maximum 38° to 39° C (G The Yeasts) It inverts saccharose, but induces no fermentation (Guilliermond *Levaduras del Pulque, Bull de la Direct des Etud biolog* 2, Mexico, 1917).

Willia anomala or Saccharomyces anomalus Hansen

(Figs 57 and 78)

This very curious species was found by Hansen in an impure brewery yeast from Bavaria It gives a rapid and vigorous fermentation in wort, and even at the beginning of the fermentation develops a dull grey film During fermentation the liquid acquires an ethereal, fruity odour (according to Seifert, ethyl acetate)

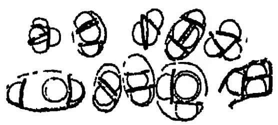

Fig 78 —Spores of *Saccharomyces anomalus* (after Hansen) —Some spores are free, others inclosed in the mother-cells On the right-hand side three spores are surrounded by the burst wall of the mother-cell

It brings about the decomposition of alcohol to form water and carbon dioxide, and finally decomposes the acetic ether According to Nielsen, it only produces 0 9 per cent by volume of alcohol in wort It ferments dextrose, but neither maltose nor lactose, and secretes scarcely any invertase Other observers have, however, found a distinct formation of invertase

The cells grown in wort are small, oval, or sometimes sausage-shaped, and in their microscopic appearance they resemble species of *Torula* When the development has gone on for some time many cells, both in the sediment and in the film, are found to contain spores

An asporogenous variety could not be developed by Hansen by cultivation at a temperature lying between the maximum for spore-formation and the maximum for budding When *S anomalus* is cultivated near the limiting temperature for budding, it grows only as sedimentary yeast The temperature limits for budding in wort are 37° to 38° C and 1° to 0 5° C

According to Will, the walls of the young cells are coloured black with 1 per cent of osmic acid This, however, does not

occur if the cells have previously been treated with alcohol. Many of the cells enclose large oil drops, which especially occur in cells derived from gypsum blocks that do not yield spores

Spores are developed on various substrata, both liquid and solid, even when abundant nutriment is present

The form of the spore is highly characteristic (Fig. 78); it resembles a hemisphere with a projecting rim round the base. On germination the spores swell and develop buds (see Fig 57)

The maximum temperature for spore-formation is 32·5° to 34° C, the minimum temperature 3° to 6° C, the optimum is 30° C (17 to 19 hours)

After Hansen had drawn attention to this curious Saccharomyces species, this or similar species were observed by many workers Thus, for instance, Meissner undertook a very comprehensive morphological and physiological investigation of three different species from Johannisberg must, from beer, and from samples of New Zealand soil He described the form of the cells (in two cases round and in the third very elongated), the appearance of the films, the fermented liquors, and the giant colonies. The pale yellow must passes gradually into a dark brown, and the liquid gives an alkaline reaction Both formation and decomposition of acid take place Reference may be made to Meissner's work on species of *Mycoderma vini* carried out at the same time

Steuber described four different kinds of *S anomalus*, three of which produce acetic ether, whilst he states that one produces both acetic ester and acetic acid Lindner gave a description of a species found in Belgian beer, *S anomalus*, var *Belgicus* or *Willia belgica* This ferments none of the known sugars, and produces no esters Lindner also discovered a species in Mazun Saito and Kozai discovered *S anomalus* in Saké Inui discovered it in Awamori, and believes that it imparts to this drink its peculiar aroma Barker found *S anomalus* by introducing ginger root into sugar solution Harrison has often found it in milk, and Holm found a *S anomalus* species in distillery mash, East Indian cane-sugar molasses, and in margarine

In English high-fermentation beers which were "fretty," the author observed a species belonging to this group, which was multiplying so freely that all other yeast-cells had been suppressed It appears distinctly as a disease-yeast causing turbidity in beer

W javanica was isolated by Groenewege from the flora of micro-organisms coating plantation rubber It has round and lengthened cells, and on glucose yeast-water quickly forms a dull, grey, deeply convoluted film Each cell contains two to four hemispherical spores. It ferments saccharose, lævulose, mannose, and raffinose, and is also endowed with the distinctive power of decomposing amygdalin into glucose, benzaldehyde, and prussic acid The

action of the emulsin is greatly enhanced by arresting the action of the living plasma. An emulsion preparation made in this manner proved superior to all other preparations. Among the carbonaceous and nitrogenous compounds assimilated cellibiose and nitrates may be mentioned

Zikes, in soil from the neighbourhood of Vienna, found a species, *W Wickmanni* (*Centr. f Bakt* 2 *abt.* 16, 1906), particularly distinguished by forming thick slimy coatings on solid media, such as wort gelatine The cell forms are very variable, sometimes lengthened Temperature limits 5° and 32° C. Spore-formation optimum 21° C. The sliminess of the cultures seems due to an excessive swelling of the cell-membrane The species assimilates glucose and fructose, and forms acetic ether

As previously mentioned, the spores of this fungus resemble those of *Endomyces decipiens,* and a relationship possibly exists between the two

Willia Saturnus or Saccharomyces Saturnus Klocker

is a species discovered in a sample of soil from the Himalayas. It forms a white film on liquids The cells are chiefly oval or round The temperature limits for budding in wort are 35° to 37° C. and 2° to 4°. The spores are lemon-shaped, with a vein running down the middle from end to end, and with a refractive granule in the centre It ferments dextrose, lævulose, raffinose, and saccharose, but neither maltose nor lactose It produces an ester during fermentation Similar species were subsequently found in samples of soil from Italy and Denmark Holm has detected it in a sample of soil from Japan

Saccharomyces acidi lactici Grotenfelt.

Grotenfelt has described under this name a species of *Saccharomyces* which, when added to sterilised milk, produces a pronounced curdling with formation of acid On gelatine and agar-agar it forms white porcelain-like colonies, and on potatoes it yields broad, moist patches of whitish-grey colour, soon turning brown. In stab-cultures in gelatine, short bottle-shaped growths develop from the line of inoculation inwards The cells are elliptical, 2·0 to 4·35 μ in length, and 1·5 to 2·9 μ in breadth

When a solution of milk-sugar is inoculated in the presence of calcium carbonate, and the product distilled, alcohol can be detected In a neutral 3 per cent solution of milk-sugar, *Saccharomyces acidi lactici* yielded 0·108 per cent. of acid in eight days

Saccharomyces fragilis Jorgensen.

While the budding and lactose-fermenting fungi found in kephir, and described by others, do not form spores, a genuine *Saccharomyces* has been found in the author's laboratory, which has been called *Saccharomyces fragilis*, on account of the feeble power of resistance of the cell-wall

The growth consists of relatively small, oval, and longish cells, with characteristic and feeble refraction At room temperature, this species behaves as a low-fermentation yeast In cultures on gypsum blocks distinct spore-formation begins in 20 hours at 25° C, and in 40 hours quite a number of free spores may be observed, at 15° C the spore-formation takes place in about 40 hours The bean or kidney shape of the spores is characteristic. Spores are also formed in growths in fermenting liquids and on gelatines, and in every case are soon set free After long standing, the growth forms a thin film, the cell-forms of which deviate comparatively little from those of the sedimentary yeast In

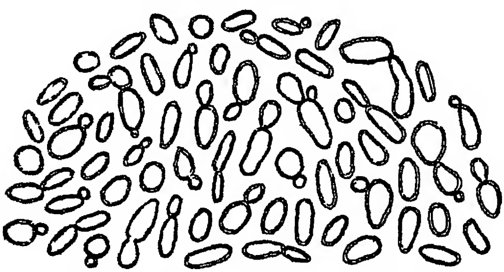

Fig. 79 —*Saccharomyces fragilis*—Young growth in lactose yeast-water (drawn by Holm from nature)

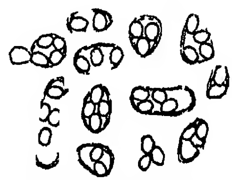

Fig 80 —*Saccharomyces fragilis*.—Spore-formation (drawn by Holm from nature).

plate-cultures the surface colonies formed in the course of two or three days at room-temperature are film-like, and liquefy, while the embedded colonies exhibit thickly-haired mycelial borders

In lactose yeast water (10 per cent.), at room temperature, this species yielded about 1 per cent by weight of alcohol in the course of eight days In two months as much as 4 per cent by weight of alcohol was produced At the same time the formation of acid began. In hopped wort (about 11 per cent Ball) it yielded at the room temperature about 1 per cent by weight of alcohol in ten days.

The optimum temperature for development lies at about 30° C.

According to Bau, this species ferments milk-sugar completely, but not melibiose Glucose is also fermented

In yeast-water glucose, lactose, and saccharose are assimilated, but not in solutions of asparagine and nutrient salts.

In "Sauer" (sour whey, used for the preparation of rennet in the manufacture of Emmenthaler cheese) Freudenreich and Orla Jensen found a Saccharomyces which produced a pleasant alcoholic odour in cream and formed spores in 23 hours at 25° C Orla Jensen isolated two species of *Saccharomyces* from Swiss butter They ferment maltose and lactose One forms spores in 24 hours at 25° C, and in three days at 30° C, the other only forms spores in six days at 30° In both cases the spore-formation is a scanty one P Mazé isolated a species directly fermenting lactose from cheese (Port du Salut), which forms spores in 24 hours at 26° C

Kuntze, in his researches on fermented milk, found a yeast in Yoghurt which plays a prominent part in the formation of aroma, producing an ester-like odour It ferments milk with formation of gas, preferably at 30° to 37° C In wort it scarcely sets up a fermentation A gypsum block culture from this medium bears some resemblance to *S capsularis*, but a detailed description has not been given

Dombrowski described two species belonging to this group *S lactis α*, isolated from Yoghurt, with ellipsoidal and lengthened cells and spherical spores, formed after 24 hours at 25° C It is a bottom-fermentation yeast, producing in milk 4·5 g of alcohol in 100 c.c In milk it sets up an active fermentation at 23° to 25° C It ferments lactose, saccharose, and glucose, not maltose *S. lactis β* from a sample of fermenting milk, shows the same cell-forms as the former At 25° C, in 20 to 24 hours, it forms one to eight spores in each cell, differing in shape elliptical, kidney-shaped, and sometimes flattened on one side Colonies on gelatine show mycelium-like off-shoots Sets up low fermentation Ferments lactose, saccharose, galactose, and glucose, not maltose It is nearly related to *S fragilis* (Comp. *Zygosacch lactis α*) *Kumys-yeast* (Rubinsky) has oval and elongated cells, and sets up bottom fermentation Neither film- nor spore-formation has been seen with certainty The fermented milk acquires a sourish aromatic smell and sourish bitter taste Ferments lactose, glucose, and saccharose In milk it forms about 0 4 per cent of lactic acid, and causes coagulation Casein and albumen are degraded to albumoses and peptones Forms ester-like bodies A somewhat similar species was formerly described by Schipin (see Kumys)

Zygosaccharomyces Barkeri Saccardo et Sydow

is a yeast species isolated by Barker from ginger It can be recognised by the fusion of two cells preceding spore-formation The development can be observed under the microscope in hanging drops of sterile water. Many of the cells will be found to produce

beak-like protuberances in twelve hours at 25° C The prolongations of two neighbouring cells grow towards each other until they come in contact At the point of contact the projections fuse together, and the protoplasm of the two cells flows together At a later stage the protoplasm separates in each cell, and two rounded corpuscles appear which become spores According to Barker, staining shows that this process is accompanied by a fusion of the cell nuclei, and Barker, therefore, pronounced it a sexual process According to Guilliermond, the conjugation of the *Zygosaccharomycetes* is exactly identical with that of *Schizosacch. Pombe* and *Schizosacch mellacei* The nuclei of the two cells fuse together always in one of the cells, and never in the conjugating passage. It may be assumed that in the formation of asci in conformity with the *Ascomycetes* an isogamous conjunction takes place

The spores swell up, and cause the walls of the mother-cell to burst, and then the spores develop in the usual way by means of budding On gypsum blocks and on gelatine the development proceeds in a similar way to that already described. The spores

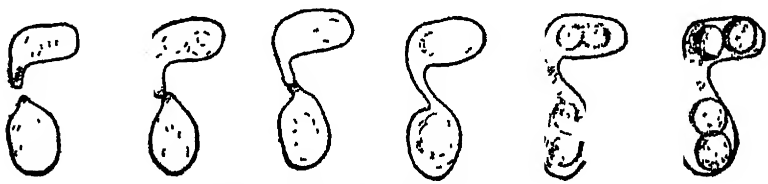

Fig 81 —*Zygosaccharomyces Barkeri*

are chiefly round, seldom oval, and rather strongly refractive. They form readily and rapidly at 25° to 30° C. The maximum temperature appears to be 37° to 38° C, and the minimum 13° C. Spore-formation may take place without fusion of the cells, as sometimes occurs in the case of *Schizosaccharomycetes* The cells are oval, no film is formed but a yeast ring develops in ten to fourteen days at 25° C The cells in the latter are oval, and occasionally elongated Spore-forming cells also occur in the ring. Colonies formed on the surface of gelatine have a smooth edge, whilst the colonies immersed in the gelatine have a fringed edge

Glucose, lævulose, and saccharose are fermented, but neither maltose, lactose, nor dextrin

Among the numerous species belonging to this genus, which seems to be very widely distributed, may be mentioned —

Two forms, "F" and "G," found by Pearse and Barker in cider "F" has oval cells The maximum temperature of budding lies between 30° and 32·5° C The spores are spherical, grouped in pairs in the copulated cells When germinating, the spore swells, and the wall of the mother-cell bursts. It ferments

saccharose, lævulose, and glucose "G" likewise has oval cells, maximum temperature of budding 32·5° C It develops readily in wort and sugar solutions The copulating cells are of irregular shape, the spherical spores are generally formed in one of the copulating cells, one or two in number It does not produce fermentation

Zygosacch lactis a, was isolated from butter by Dombrowski Its spherical cells show numerous fusions; from one to four spherical spores are formed in both, or only one, of the cells fused together, they are soon set free It is a low-fermentation yeast: In 5½ months 4·5 g. alcohol per 100 c c of must was produced. It ferments lactose, saccharose, dextrose, and galactose, but not maltose.

Zygosacch Priorianus (*Lafar's Handbuch IV*) was found by Klocker in honey-bees The cells differ in shape, they are intimately connected, and form a rather compact growth on the bottom of the flasks They attain their largest size at 13° to 16° C Temperature limits for the growth in wort 3° to 8° and 36° to 38° C The colonies are smooth at high temperatures, otherwise wrinkled or folded Spores round or oval, free formation on wort-gelatine and slices of carrot It ferments glucose and maltose, not saccharose and lactose.

Z Javanicus was found in Java by Kryff It is a low-fermentation yeast, and ferments saccharose, glucose, lævulose, and galactose The ellipsoidal cells have their optimum for budding at 34° to 35° C in wort Abundant sporulation on agar

Debaromyces globosus was found by Klocker on soil from the island of St Thomas. The cells in wort are spherical The spores (usually one in each cell) are uniform, their surface is covered by very small excrescences, they develop freely on gypsum blocks, the temperature limits being 14° and 34° to 36° C According to Guilliermond spore-formation is sometimes preceded by copulation between two equal cells or between a full grown cell and a disengaged bud The nuclei of the two cells may fuse together in the connecting channel and then divide, each nucleus passing into its own cell and forming a spore, or else after fusion the nucleus may enter one of the cells and form a spore This species develops rather quickly in wort at 25° C It ferments dextrose, inverts saccharose and ferments invert-sugar, but does not ferment maltose

D tyrocola, found by Konokotina in cheese, shows a copulation process similar to that of the preceding species It inverts saccharose, but does not produce fermentation

Schwanniomyces occidentalis, likewise found by Klocker in soil from St. Thomas, is allied to the same group. The cells are elliptical at or about 8° C, and spherical about 36° C Guilliermond observed cells with beak-shaped prolongations and also cases

where two prolongations met without a distinct copulation taking place. The spores (mostly one to a cell) take the form of a flattened sphere, surrounded by a band, the surface being covered by small excrescences They develop readily on gypsum blocks at 28° to 32° C. The species grows slowly in wort Ferments dextrose, but not maltose nor lactose An aqueous solution of saccharose is slowly inverted and weakly fermented, but if yeast-water is added to the solution inversion takes place quickly.

Guilliermondia (Nadsonia), fulvescens, detected by Nadson and Konokotina in oak mucilage, has oval, elliptical and lemon-shaped cells On meat-pepton-gelatine with $\frac{1}{4}$ per cent. glucose the following development will take place most readily —A full grown cell puts forth from its narrow extremity from one to four small buds, these drop off, except one which amalgamates with the mother-cell through a connecting channel From the opposite extremity of the original mother-cell, a bud is susequently formed, in which appears a spherical spore, the plasma of the two copulating cells passing into the spore-cell. The spore has a thick brownish-yellow membrane, dotted with small excrescences. Upon gelatine the spore cultures are, therefore, yellowish or reddish-brown, whereas the vegetative cultures are white Asporogenous growths sometimes occur The spore cultures liquefy gelatine. The species ferments glucose, fructose, galactose, saccharose, and maltose

G. elongata, found by Konokotina in a gummy exudation on birch trees, is distinguished from the previous species by the fact that it ferments glucose and lævulose, but not saccharose, maltose, lactose, nor galactose

A similar copulation between a full grown cell and a bud ("Heterogami") was observed by Cesare and Guilliermond in a number of forms found as white spots on sausages, corned meat, and the like The development of the spores (coated with a rough membrane) was observed on slices of potato and carrot, and on Gorodkowa's gelatine (water 100, gelatine 1, meat juice 1, sodium chloride 0·5, glucose 0·25), but not in gypsum cultures. Conjugating cells were often found in the same colony, originating from one cell. In general, only one spore is formed in each ascus Optimum about 25° C Some of these species developed as sedimentary yeast, others as films The growths on gelatine and potatoes acquire a brown colour. These forms invert saccharose, but do not produce fermentation

Zygosacch Chevalieri was isolated from wine by Guilliermond. The round, oval, or lengthened cells form a slimy film and abundant deposit in saccharine liquids Spores are formed both in the film and on solid media, they are semi-globular with a bulging of the flat side, and bear a close resemblance to those of *Pichia membranæfaciens* They are generally formed by fusion of two cells of unequal size. The smaller cell, a young detached bud, unites

through the medium of a canal with a full grown cell, the two nuclei meet in the canal, merge into each other, and migrate into the big cell, where one to four spores are formed The germinating spores can also copulate, a new spore may be formed in the interior of another. This species does not produce fermentation in wort or saccharose, nor in any other of the common sugars, but it inverts saccharose

Z. Nadsonii was found by Guilliermond in orange syrup. It quickly forms a deposit and a film at 25° to 30° C The cells are round and oval, torula-like, and afterwards become lengthened, particularly at the limit of temperature 41° to 42° C. Conjugation takes place between a mother-cell and an unripe bud, which send forth prolongations towards each other The optimum for spore-formation lies between 23° and 30° C The spores are of irregular shape, one side flat, the other tapering This species inverts saccharose, and ferments invert sugar, also glucose and lævulose

Z Mandshuricus was found by Saito in the so-called Chiidzu or Chinese yeast It is related to *Z Barkeri* in its development In the cells are formed from one to four hyaline spherical spores, which also occur in sedimentary yeast in wort. On gelatine there appears an asporogenous variety the gelatine is not liquefied. It ferments dextrose, lævulose, mannose, saccharose, and raffinose, but not maltose and lactose

Z. major, isolated by Takahashi, appears to be active in soy fermentation It has spherical cells (3 to 7 μ), which in the yeast ring are very lengthened It is a low-fermentation yeast Very resistant to common salt. will grow in koji extract with 20 per cent salt Ferments saccharose, maltose, glucose, lævulose, and mannose Spores readily developed in yeast ring on very dilute soy, but not on gypsum blocks

Z Soja is stated by Takahashi to resemble Saito's *Sacch. Soja*, which is probably a Zygosaccharomyces It does not ferment galactose, but maltose, dextrose, lævulose, and mannose Like the preceding species, it forms plentiful spores under similar conditions.

On the same material Saito observed a film-forming species, *Z Japonicus*, with round cells in a young state and round and oval spores. It ferments maltose, glucose, and lævulose Spores were developed by growing the cells in dilute soy, whence they were transferred, from the yeast ring, to sterilised water in a Bottcher's chamber They were also developed on gypsum blocks with 0·5 per cent of dextrose and 4 to 10 per cent common salt

A similar species, *Z salsus*, described by Takahashi, forms a thick corrugated film on soy and koji extract containing a quantity of salt

Z mellis acidi was found by Richter in comb honey It ferments the honey (about 80 per cent sugar), and has very

small (3 to 4 μ) spherical or slightly ellipsoidal cells, copulating and forming globular spores in both cells. It ferments glucose, fructose, and saccharose, also galactose, but weakly, and forms acids.

On cankerous spots of *Opuntia ficus Indica*, Ciferri detected a zygosaccharomycete producing very weak fermentation in unfermented wine The cells form chains consisting of three to five cells By copulation this species forms asci containing two to four oval or round spores with a thin membrane On gelatine white, later yellowish-white, colonies are formed with curved serrated edges; radial and concentric lines

Saccharomycodes Ludwigii or Saccharomyces Ludwigii Hansen. (Figs 55, 56, and 82.)

This remarkable species, which was discovered by Ludwig in the viscous secretion of the living oak, is the only one of the known *Saccahromycetes* which can be recognised solely by means of a microscopic examination The following description is taken from Hansen's investigations The cells are very variable in size, elliptical, bottle-shaped, sausage- or frequently lemon-shaped. Partition walls may occur in all the complex cell-combinations. The vegetative growths in wort-gelatine are round like those of the majority of the *Saccharomycetes*, and are either pale grey, or faintly yellow In wort, it yields only 1·2 per cent. by volume of alcohol, even after a long-continued fermentation; and this accords with the fact that maltose is not fermented by this species. In dextrose solutions, on the other hand, it yields up to 10 per cent. by volume of alcohol. It inverts saccharose, but does not ferment solutions of lactose nor dextrin, neither does it saccharify starch paste It assimilates glucose and saccharose in yeast-water, also in asparagine and peptone solutions with nutrient salts It readily develops spores in aqueous solutions of saccharose, in wort-gelatine, in yeast-water, and in wort, in the latter case, even when no film has formed

It is characteristic of this species that a fusion of germinated spores often occurs, especially in the case of young spores, and these new formations develop germ-filaments (promycelium), from which new yeast cells are gradually marked off by sharp transverse septa (Figs 55, 56). At the ends of these cells, buds develop, and these again are marked off by transverse septa.

According to Hansen, a stable asporogenous variety cannot be prepared by cultivation at temperatures lying between the maximum for spore-formation and the maximum for budding. On the other hand, Hansen found that a number of cells lost their power of forming spores when they remained for some time on one and the same substratum In other cells this power was diminished, and the remainder do not appear to be influenced.

By cultivation in favourable liquids the first set of cells again yield spores Beijerinck found that the colonies formed from asporogenous cells do not liquefy gelatine, which is contrary to the custom of sporogenous colonies.

Guilliermond, who also examined the germination of spores, records amongst other facts that spores which are not derived from the same ascus may fuse together, and further that the spores which grow together by fusion may then develop to asci with spores, without previously forming new cells He finally showed that by the fusion and germination of spores a combination of their cell nuclei and a subsequent division take place as soon as the promycelium has reached a certain size Guilliermond described this process as conjunction with isogamy

Whilst in the case of *Schizosaccharomycetes* and *Zygosaccharomycetes* a conjunction takes place between the vegetative yeasts, converting these into an ascus, in this case conjunction takes place between the spores and even before they germinate.

Will discovered that the spores may sometimes be more or less distinctly stained to a bluish-green with iodine in potassium iodide (a similar colour to that given by *Bact Pasteur. Hansen*).

In old cultures (Fig 82) there is a strong tendency to form mycelia, but it is only exceptionally that portions are found where the links are closely bound together and only display slight contractions. Such portions are provided with distinct and straight septa. Every cell of such a colony can form spores as well as buds Amongst them odd shapes and very large, strongly branched cells are found It is characteristic of this species that the cells die off within two years in aqueous sugar solutions, whereas they can keep alive for many years in beer-wort The temperature limits for budding in wort are 37° to 38° C and 3° to 1° C The maximum temperature for spore-formation is 32° to 32·5° C, the minimum 3° to 6° C, the optimum 30° to 31° C. (18 to 20 hours).

A variety of *S Ludwigii* was detected in oversulphured wine-musts by Kroemer and Heinrich, who found it to be characterised by the presence of big granules in the cells It forms plenty of subspherical and polyhedral spores. Ferments glucose, fructose, galactose, mannose, saccharose, and raffinose In must the fermentation is sluggish A content of 470 g. SO_2 per litre of must is unable to check the growth The upper limit of growth is at 37° C, the upper temperature limit 54° C

From Mexican Pulque Guilliermond (*The Yeasts* 1920) isolated a closely allied species, which inverts and ferments saccharose. It develops a vigorous, typical mycelium, budding like *monilia*, and forms plenty of spores in the mycelium and yeast cells on solid media The spores during germination behave like those of *S Ludwigii*; immediately after fusion new spores fairly frequently form inside those already present.

An allied species, *Sacch. paradoxa*, was observed by A. Bartschinskaja in the gummy exudation of oak and elm. The germinating spores copulate and form a promycelium. Promycelia of different spore-associations may further copulate, and the buds of copulating mycelia also sometimes copulate two by two. The various fusions are followed by spore-formation in all the cells,

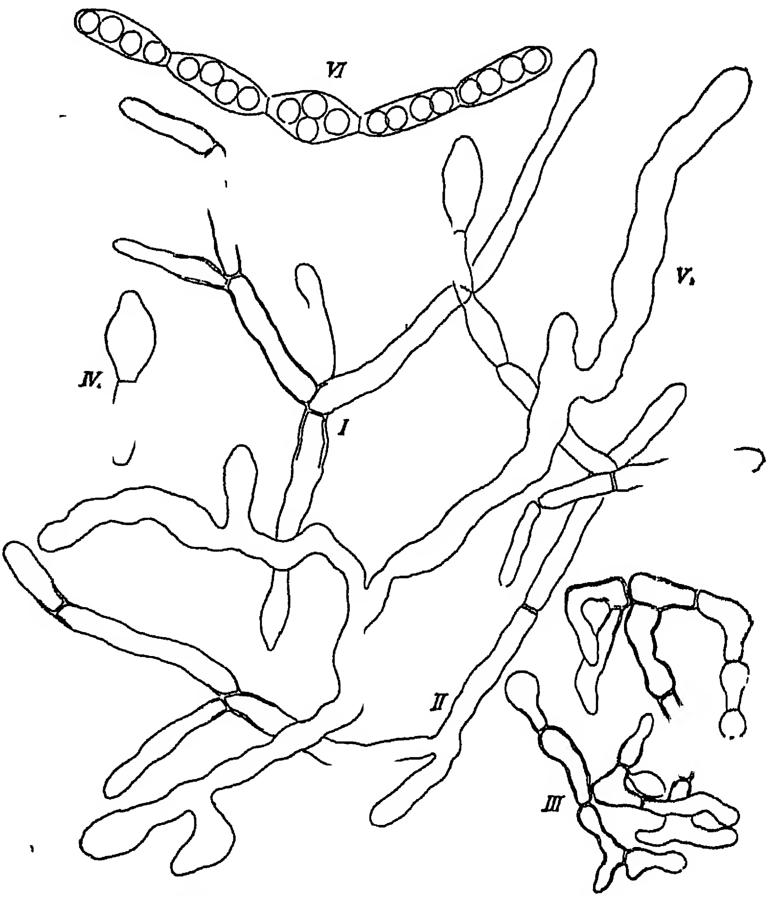

Fig 82 —*Saccharomyces Ludwigii* —Old film and mycelium (after Hansen).

even in the copulating spores Resting cells may occur in the different generations. It ferments glucose, lævulose, saccharose, and galactose

A series of recently discovered species closely allied to *S. Ludwigii*—the *Schizosaccharomyces*—are distinguished by the total disappearance of budding, and the fact that the propagation of the cells takes place by division.

Schizosaccharomyces or Saccharomyces comesii Cavara

was described in 1893 It lives as a parasite or saprophyte on the sheaths or pedicles of millet, and, according to Cavara, forms a mycelium consisting of cylindrical hyphæ with partition walls; this mycelium produces cylindrical or longish ellipsoidal conidia, 7 to 8 μ long and 2 to 3 μ broad, isolated or linked together In sugar solutions it produces a growth of yeast, and when the nutritive solution is exhausted, spores appear within the cells These spores are globular, two to four in each cell Two or more fuse together in the mother-cell, through the membrane of which the germinal threads appear

Schizosaccharomyces (Sacch.) octosporus

was discovered by Beijerinck on dried currants, and has been more recently examined in the author's laboratory by Schionning in growths on raisins The propagation takes place in the following manner (Fig. 83) About the middle of the cell a partition-wall

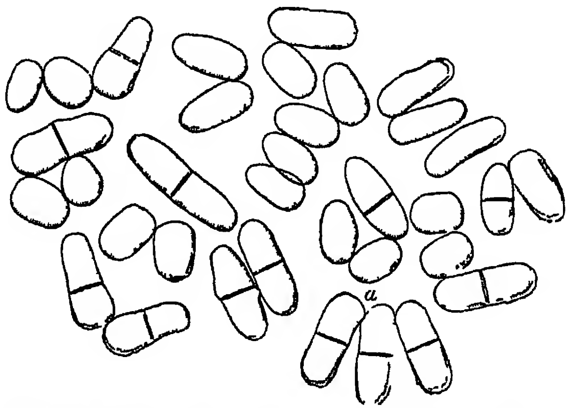

Fig. 83 —*Saccharomyces octosporus* —Young growth after cultivation for twenty-four hours in beer-wort at 25° C (after Schionning)

appears, after this has split up, the two new cells assume a round shape, and revolve round a point of the septum, where connection is still maintained, so that at last they lie almost parallel Finally, they separate entirely from each other, having taken an ellipsoidal or oval shape, they then extend in length, and the division begins afresh. But it may also occur that two cells, still connected throughout the full extent of the partition-wall, increase in length and form fresh septa, so that the original mother-cell appears divided into four or more cells The cells are 4·5 to 6 μ broad and 7 to 13 μ long Even at the beginning of the fermentation in wort at 25° C the cells form endospores; but the spore-formation is very feeble both under ordinary fermentative conditions

in wort and also during cultivation on moist gypsum blocks. Seiter found that the spores are formed on gypsum blocks in six to seven hours at 25° C This development is much more vigorous on the surface of nutrient gelatines, such as wort-gelatine (Fig. 85), where it forms round, waxy, and raised colonies with a depression in the centre The cells grow shorter and more rounded after developing for some days at the temperature of the room, and the ascus-formation, according to Schionning's observations in the Carlsberg laboratory, now takes place as follows (Fig. 84) :—

The rounded cell lengthens, a partition-wall appears, which splits off, after which the two new cells merely touch or connect at one point. They then, again, coalesce, and at last form a

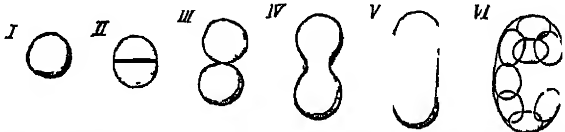

Fig 84 —*Saccharomyces octosporus* —Development of the ascus (after Schionning).

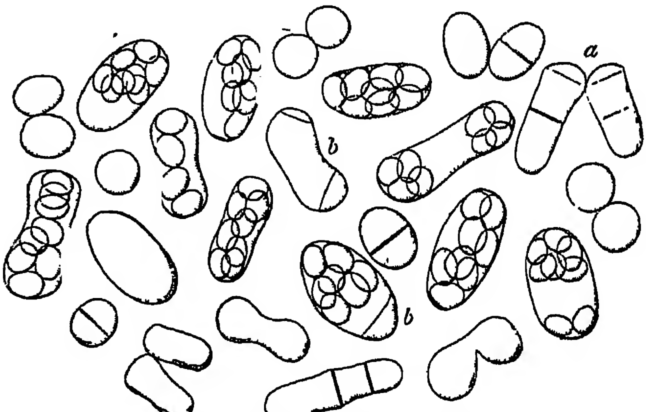

Fig 85.—*Saccharomyces octosporus* —Young growth in wort-gelatine (after Schionning).

lengthened, ellipsoidal, hour-glass shaped or irregular cell, which gradually increases in bulk (frequently 14 to 20 μ long) In these cells eight spores form, as a rule, but frequently only four, and less frequently from two to seven By degrees the wall of the mother-cell dissolves, and the spores now lie embedded in slime, which subsequently disappears. The spores are often oval, and, according to Lindner, their membrane is coloured blue by a solution of iodine in potassium iodide According to Guilliermond, the ascus-formation may also proceed in this way Two cells which have not been derived from one and the same cell fuse together, or again an ascus may be formed without the fusion of two cells.

Both on the vegetative cells and on the asci of the spores fine

lines may sometimes be observed, which form the limit between the older, thicker parts of the cell-wall and the newly-formed, thinner parts The latter appear after the partition-walls, which now form the terminal walls of the new cells, have divided, or after fusion, through the ensuing growth of the ascus.

On wort no film has been observed , only a slender yeast-ring.

Wort-gelatine is rapidly liquefied by *S octosporus* Beijerinck found that in the case of the asporogenous cells the formation of trypsin is considerably reduced, while the formation of acid is greater than in the sporogenous cells

This species ferments maltose, lævulose, and glucose, but it does not invert saccharose According to Fischer, an aqueous extract of the dried pulverised growth decomposes maltose, but does not exert any influence upon saccharose He states that it contains raffinase, and is, therefore, capable of fermenting raffinose.

It assimilates glucose and maltose in yeast-water and in solutions of asparagine and nutrient salts (Klocker)

Schizosaccharomyces (Sacch.) Pombe

was discovered by Saare and Zeidler in millet beer from Africa, and more exactly described by Lindner It is closely allied to the previous species , its propagation also takes place by formation of partition-walls and by fission , frequently the two new cells remain connected for some time at a single point, upon which they rotate until they form an acute angle to each other The cells resemble the conidia of *Oidium* , but the shape of many of them is suggestive of the manner in which they were derived, one end being rounded, whilst the other is surrounded by a well-defined ring-wall, enclosing the newly-formed piece of globular membrane. In the cells one to four spores may occur, which grow in the same way as those of *S Ludwigii*—viz , by the formation of a germinative thread , no fusion of the promycelium of the spores has been observed

Guilliermond has closely examined the course of spore-formation, and finds that the ascus-formation often follows a fusion of two cells which may be sister cells In this way the dumb-bell shaped cells often met with in spore-cultures are formed. He also observed a fusion of three cells Spores also form in the sedimentary yeast By germination they swell up and form a germinal tube, which afterwards divides into two cells by means of a septum

The growth forms no film on wort On wort-gelatine it forms a compact finely-furrowed growth

At its optimum temperature, 30° to 35° C , this species shows high-fermentation phenomena It is distinguished by the considerable amount of acid formed during the fermentation, and

possesses a certain power of resistance in competition with bacteria. In beer-wort it gives a rather vigorous fermentation; it also produces fermentation in dextrose, maltose, and cane-sugar solutions It does not ferment *d*-mannose, but does ferment dextrin

According to Rothenbach's experiments, it ferments about half the total amount of diastase-dextrin prepared according to Lintner's directions, leaving achroo-dextrin, which, on addition of alcohol, slowly separates out in sphæro-crystals

As this species is capable of forming very considerable amounts of alcohol, it might be supposed to be of industrial value Experiments made in this direction, however, have hitherto proved unsuccessful.

Lepeschkin often found mycelial formations in young cultures of *S Pombe.* This formation remained through countless generations He believes that the mycelium is not to be regarded as a normal form of development, but only occurs through the reconstruction of cells The requisite conditions are unknown.

Nakazawa (Ueber Garungsorganismen von Formosa, *Ber. d. Inst fur Exper. Forsch.*, 1914), in sugar products from Formosa, met with some similar species, *S Formoensis*, *S Sautavensis*, and *S nokkoensis*, the last being distinguished by the fact that the spore-formation is not preceded by a copulation, and that the spores differ in shape . spherical, ellipsoidal, and hemispherical.

In the rum-fermentation of molasses in the West Indian Islands, two different yeast types occur In a few districts the common ellipsoidal form predominates, in other districts a mould-like *Saccharomyces* In arrack-fermentation of molasses in Java, Vordermann and Eykmann constantly found a fungus which separates new cells through formation of partition-walls; no spore-formation was observed, and, according to Eykmann, the fungus recalls *Hyphomycetes* in its growth forms A Saccharomyces of similar appearance was discovered by P Greg while working in the writer's laboratory, in cane-sugar molasses as used in rum-fermentation in Jamaica This is designated —

Schizosaccharomyces or Saccharomyces mellacei Jorgensen. (Figs 86 and 87.)

In cylindrical vessels at 25° C this species ferments beer-wort with top-fermentation phenomena, forming a caseous, loose deposit During fermentation it develops a pleasant aroma. In wort of 10 5 per cent Ball. it produces about 2½ per cent. by weight of alcohol

The different forms assumed by the species, recall *Saccharomyces octosporus*, *Sacch Pombe*, etc In old cultures very curious cell-forms (Figs. 86, 87) occur, which also develop during fer-

mentation. In wort-cultures five months old no film had developed; only a yeast ring was observed The liquor is not decolourised by old cultures

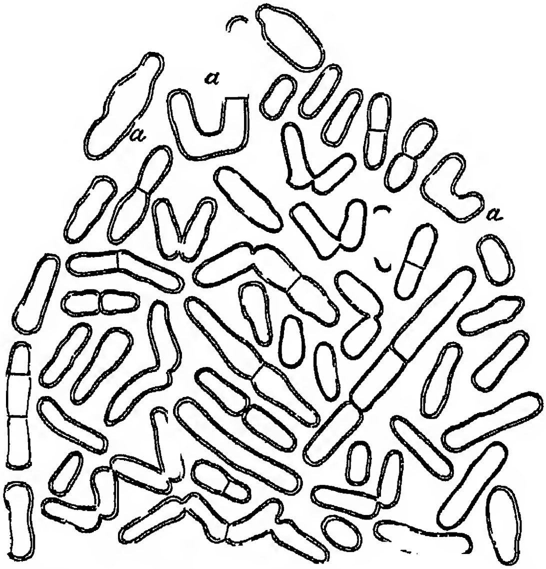

Fig 86.—*Saccharomyces mellacei* —Young culture in beer-wort, *a*, cells after eight days' cultivation (drawn by Holm from nature)

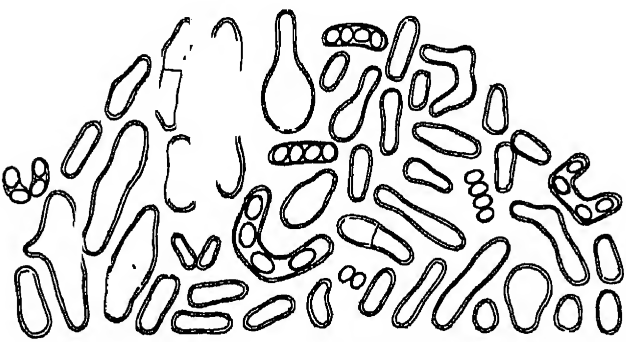

Fig 87 —*Saccharomyces mellacei* —Growth from the yeast ring in beer-wort (drawn by Holm from nature)

The spores (Fig 87) are oval They occur in all cell-forms, generally four to the cell, they refract light strongly, and, according to Holm, they are coloured blue by iodine

Guilliermond found that an ascus is produced with this species by the fusion of two cells which are often sister cells, but ascus-formation may take place without previous fusion

In plate and streak-cultures the growths, both on and below

the surface, have a sharp-cut edge, the cell-forms are similar to those in liquids, cells shaped like the conidia of *Oidium lactis* frequently occur

It ferments dextrose, maltose, and saccharose, as well as *d*-mannose and dextrin According to Lepeschkin, *S mellacei* may form a mycelium.

According to investigations made by P Greg in the author's laboratory, divergencies of a marked and permanent character distinguished the species belonging to this type Thus some yield malodorous products in the fermenting liquor, some very fine products, whilst others differ greatly in the length of time required to complete the fermentation in one and the same sterilised molasses and dunder under identical conditions. The amount of alcohol produced by these types varied from 6 6 to 7 6 per cent. by volume. The rate of multiplying also differed widely in these races Further details relating to comparative results in practice are given in papers by Greg as well as by Hart, who has carried out rum-fermentation with ellipsoidal species (Botanical Department, Trinidad).

Thorough researches on rum-fermentation in Jamaica were carried out more recently by Allan and Ashby, who described the various active organisms There is a well marked difference between clean rum and flavoured rum In the manufacture of the former, with comparatively low acidity, there occur both ellipsoidal or oval and fission yeasts, the ellipsoidal forms sometimes predominating In flavoured rum, where the acidity is very high, the budding species are repressed or entirely suppressed by the fission-yeasts, which alone are able to support such high acidities The latter are produced in a peculiar fermentation, where a mixture of dunder (the residue of distillation), containing a large quantity of dead yeast, wash and mash (the crushed sugar-cane after extraction of the juice), enters into bacterial fermentation, gradually resulting in an abundant formation of acetic, lactic, and butyric acids, brought about by many different species of bacteria The "flavour" thus produced is added to the wash in the large vessels after fermentation has begun The alcohol formed by the fermentation combines with the various acids to form compound ethers, which impart a special character to the flavoured rum Among the different varieties of *S mellacei* the slow working top yeasts are to be preferred because, during the slower fermentation, there is more time to complete the chemical reactions, the bottom-yeast varieties examined would bring fermentation too rapidly to a close Both types produce very large amounts of alcohol Various torula and anomalus forms make their appearance during these fermentations

More recent investigations by Kayser, who used the different species isolated from spontaneous fermentation, in parallel for-

mentations in sterilised molasses, confirmed the results obtained in the first experiments made in the author's laboratory, and proved once more that the yeasts concerned are directly responsible for the formation of ethereal bodies in the finished product, and that among these budding fungi, schizomycetes and film-forming species, there exist a number of races producing varying amounts of alcohol, volatile and non-volatile acids. By adopting the most suitable race, the fermentation may be shortened considerably, and a uniform product obtained. Moreover, the amount of volatile and non-volatile acids is dependent on the conditions under which the micro-organisms develop—supply of air, temperature, and composition of the medium. A more copious supply of air will effect a change in the relative proportion of fixed and volatile acids where certain races are concerned, but not in the case of others. Some races, in the course of fermentation, form more butyric acid at 25° C than at 35° C, on addition of ammonium sulphate (8·6 pro mille) to the molasses, a budding species gave a very considerable increase of volatile oils, but a schizosaccharomyces, a decrease. On addition of dunder to the molasses, the amount of volatile acids was considerably increased, as proved also by Allan's and Ashby's researches. The same result was obtained by letting one of the bacteria isolated co-operate with the yeast. Thus, all the investigations go to prove that it is the right selection of the active micro-organisms that decides the quality of the product.

Saccharomycopsis guttulatus or Saccharomyces guttulatus (Robin) Wilhelmi

was found by Remack in 1845 in the contents of the stomach and intestines of a rabbit, and subsequently described by Robin under the name *Cryptococcus guttulatus*. Both classified it amongst the yeast fungi. In 1896 Buscalioni gave a comprehensive morphological description of the organism, which he named *S. guttulatus*. We are indebted to Wilhelmi for the following description (Schionning has associated this form with one described by him as *Saccharomycopsis capsularis*). The cells are elliptical and longish-oval with blunt ends. The length varies from 6 to 16 μ, width 2 to 4 μ. They contain abundant quantities of glycogen. With poor nourishment, from two to four large and strongly-refracted vacuoles are observed. Budding is linear or spiral. Under favourable conditions of nutriment the buds are detached at an early stage of development, if the

Fig 88 —*Saccharomycopsis guttulatus*

conditions are unfavourable, bushy colonies are formed In the mother-cell from two to four elongated oval spores are formed, provided with two membranes (exosporium and endosporium) (Fig 88)

On germinating, the exosporium bursts near one of the poles, or at the side, always with an irregular edge, and crumples up to form a small indistinct residue, which usually clings to one end of the endosporium Germination and growth of the spores take place in presence of from 1·25 to 5 per cent of hydrochloric acid and 10 per cent. of sugar, at a temperature of 37° C., whilst spores are formed at a temperature of 14° C.

Saccharomycopsis capsularis Schionning

was discovered in an analysis of soil. The sample was taken from the neighbourhood of the St Gotthard Pass The youngest sedimentary growths in beer-wort consist of variously shaped cells, especially *Pastorianus* forms, often having pointed ends Within two days small islands of a film appear on the surface of the liquid, consisting of typical-branched mycelium with septa, partly breaking into bud and partly separating into round or *Oidium*-like links At a later stage a few mycelial forms are found in the sedimentary yeast The covering becomes thick and uneven with a dry, white, and slightly tufted appearance In those surface cultures a few spores occur later, especially in the round cells or *Oidium*-like cells formed at the end of the threads, but occasionally in the mycelial threads themselves These spore-forming cells contain a specially bright refractive protoplasm; four spores almost always form in each ascus They are coloured bright rosy-red with concentrated sulphuric acid The spores have a flattened spherical shape surrounded by a fine transverse line. During germination it can be seen that the spores are provided with a double wall (exosporium and endosporium). The outer opens up into two unequal valves and divides along the transverse line. The two valves are fastened together at one point, and lie like a pair of mussel shells round the swollen cells, which soon begin to bud It is the exosporium that gives a red coloration with sulphuric acid and other mineral acids. It probably contains cork.

On the surface of wort-gelatine agar greyish-white colonies form with a slightly hairy appearance. They gradually change to a chocolate-brown colour. On gypsum blocks spore-formation is not so abundant as on solid yeast water substrata

It ferments maltose, dextrose, lævulose, and *d*-galactose, but not *l*-arabinose, raffinose, lactose, or saccharose saccharose is also not inverted In ordinary beer-wort (about 13·3 per cent. Balling) it can produce 7 per cent by volume of alcohol

Optimum temperature for vegetative growth 25° to 28° C, maximum temperature 38·5° C, minimum under 0 5° C Optimum temperature for spore-formation 25° to 28° C, maximum 34 5° to 35° C, minimum between 5° and 8° C *

II Budding Fungi without Spore-formation.

Torula.

These yeast-like forms were first characterised by Hansen. They are widely distributed, and, therefore, not infrequently occur in physiological analyses connected with fermentation They occur in both spherical and more or less elongated forms, and are distinguished from the genus *Saccharomyces*, as first pointed out by Hansen, by their inability to form endogenous spores In most cases they multiply only by budding, in some few cases also by the formation of mycelium.

According to the author's researches, certain *Torula* species may act as disease-yeast, for they multiply freely and give rise to a kind of turbidity in weakly fermented, high-fermentation beers, when these are bottled, the character of this turbidity, however, is somewhat different from that caused in low-fermentation beer by the wild *Saccharomycetes*

In sugar-works, the writer finds *Torula* species occurring extensively, frequently in large quantities, even in the finished product Among the species examined many possess an inverting enzyme It is not improbable that these growths assist in the progressive formation of invert-sugar which frequently takes place during the storage of cane-sugar

Hansen has observed many different species, and has described the following in detail —

The *first* occurs in wort, the cells being either single or in small clusters. Some cells have a large vacuole in the middle, and this sometimes contains a small strongly-refractive particle The size of the cells varies considerably (1·5 to 4·5 μ). This species does not secrete invertase, and causes a scarcely perceptible alcoholic fermentation in beer-wort.

Under the same conditions the *second species* possesses larger cells than the first (3 to 8 μ); they resemble the foregoing, except that the contents of the cells grown in wort are often very granular

The *third species* which, microscopically, resembles the last, produces under the same conditions as much as 0·88 per cent

* Certain dubious species of Saccharomycetes must be mentioned, one isolated by Metschnikoff, *Monospora cuspidata*, occurring as a parasite in *Daphnia*, which has long cells and contains one needle-shaped spore, and another, *Nematospora Coryli*, isolated by Peglion from hazel nuts, containing eight spores to a cell, in two bundles, each of four spores. The spores carry a long flagellum at one end, which disappears before the germination of the spore takes place.

by volume of alcohol, it gives a distinct head with evolution of carbon dioxide, but it cannot invert cane-sugar

The *fourth species* (2 to 6 μ) inverts cane-sugar and produces slightly more than 1 per cent by volume of alcohol in wort with considerable frothing, it does not, however, ferment maltose

The *fifth species*, which in the form and size of its cells resembles the first, develops a uniform, dull grey film on wort and yeast-water at the ordinary room temperature, likewise on lager beer, and even on liquids containing as much as 10 per cent. of alcohol. It inverts cane-sugar, and forms a slight film on the solution It does not, however, excite any appreciable alcoholic fermentation

A *sixth species* (Fig 89), which forms spherical and oval cells, gives a distinct fermentation in beer-wort, yielding as much as 1·3 per cent by volume of alcohol. It does not ferment maltose solutions. It inverts cane-sugar, and in 10 per cent. and 15 per cent solutions of this sugar in yeast-water, it yields respectively 5·1 and 6·2 per cent by volume of alcohol, after fourteen days at 25° C.; the latter culture gave 7 per cent by volume of alcohol in two months Dextrose solutions of the same concentration and under similar conditions gave 6·6 and 8·5 per cent. of alcohol by volume

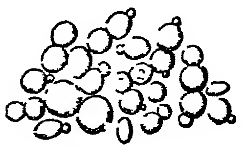

Fig 89 —*Torula* (after Hansen) —Sedimentary forms after one day's cultivation in beer-wort at 25° C.

The *seventh species* (Figs 90 and 91) was found in the soil under vines. The sedimentary cells are most frequently oval and in part larger than those of the last species Certain cells of the films are very irregular in shape This *Torula* produces only 1 per cent by volume of alcohol in wort, does not ferment maltose, and neither ferments nor inverts cane-sugar In 10 per cent. and 15 per cent solutions of dextrose in yeast-water it gave 4·6 and 4·5 per cent by volume of alcohol in 15 days at 25° C., and 4·8 and 4·7 per cent in 28 days In two other flasks 4·8 and 5·3 per cent of alcohol were produced after long standing Hansen assumes that species such as the sixth and seventh, which produce a vigorous fermentation in dextrose solutions, take part in the fermentation of grape and other fruit juices. On the other hand, they have probably little importance in breweries and distilleries, since they are unable to ferment maltose

The most extensive researches on torula species, particularly on those occurring in *breweries*, were carried out over a period of years by Will He showed how widespread they are in that industry, where most of them, however, do not exert any injurious influence on the product After a thorough-going study of a number of species, both morphologically and physiologically,

he divides this class of organisms into two groups, comprising a number of growths or species, the distinctive characters of which are not, however, always well marked, even under identical external conditions Some difficulty may therefore be experienced in identifying any given species with one of those described The following is, in its main features, the characterisation given by Will of those two principal groups and the forms belonging to them —

The *first group*, including the two genera *Eutorula* and *Torula*, is distinguished by the fact that under most conditions it appears as spherical or sometimes slightly ellipsoidal cells of different sizes in the different varieties, but of fairly equal size within each

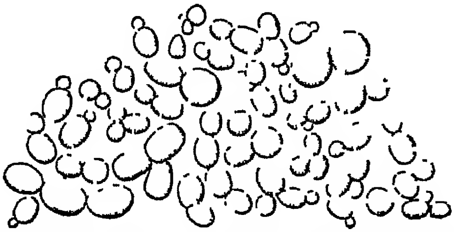

Fig. 90 —*Torula* (after Hansen) —Sedimentary forms after one day's growth in beer-wort at 25° C

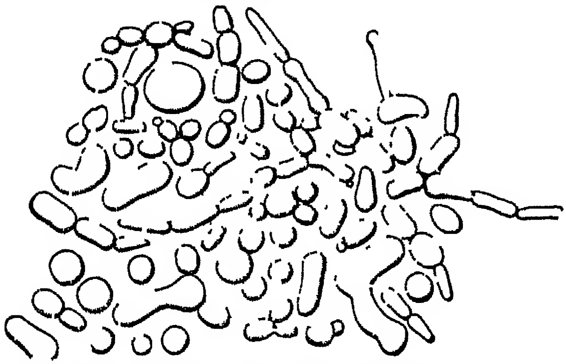

Fig 91 —*Torula* (after Hansen) —Same species as Fig 90 Film formation on a wort-culture ten months old

of these In some varieties the cells are as large as those of beer yeast, whilst in others they are so small as to approach the size of spherical bacteria, lengthened cell-forms are very rare To this group belong the various Hansen species described above, and also the following cultures described by Will, which are likewise aerophilous organisms :—

Eutorula vulgaris includes widely distributed varieties of frequent occurrence The characteristics common to them are the general aspect of the cells, and, when propagated in large cultures, a formation of films and giant colonies, also their power of producing colouring matter The cells are spherical to slightly

ellipsoidal, of varying size, with one or two small oil-drops in a slightly refractive plasma Budding in unbranched series, or in the same succession as in saccharomycetes Velocity of film-formation varying in different cultures, the islets as soon as formed merge into a coherent film, which, unless it sinks to the bottom, grows into a thin surface coating with mesenteric creases, sometimes creeping up the sides of the glass The single cell colonies embedded in a solid medium are lenticular or spherical, while the superficial colonies are flat with a more or less irregular growth The giant colonies on wort-gelatine spread over the surface, forming radial creases or stripes, or building up "ramparts"; the border zone is sometimes lobate with a wavy edge, and the gelatine is liquefied The varieties react differently on the sugars some of them ferment maltose and lactose, others do not, but all of them ferment dextrose, lævulose, galactose, and saccharose They assimilate citric, malic, succinic, and lactic acids, also alcohol They all produce a *colouring matter* imparting a more or less marked green or yellowish-green colour to the liquid.

The temperature limits of growth vary in the different varieties; for instance, in hopped wort, the upper limit fluctuates between 30° C for some varieties, and 38° C for others.

Eutorula ellipsoidea is identical in most respects with the preceding species, but is distinguished by the more frequent occurrence of ellipsoidal and somewhat tapering cells, and at favourable temperatures budding associations are formed of elongated sausage-shaped cells Further, the giant colonies exhibit grape-like clusters on the under side, whereas the preceding species give tight bundles On the surface of liquid media a thin film with a rather marked ring slowly develops The species is characterised by a relatively high fermenting power, producing well-marked fermentation phenomena, particularly in the case of saccharose; dextrose, lævulose, galactose, maltose, and raffinose are also fermented

The following are cognate forms —

(*Torula*) *Eutorula rubra*, which, with the next species, was described by Schimon It forms a light-red colouring matter (carotin); old cultures in wort exhibit yellow oily drops Cells ellipsoidal, with slimy membrane, in old cultures causing ropiness. Assimilates dextrose, lævulose, galactose, and saccharose, to a less extent lactose; does not ferment any of the sugars.

(*Torula*) *Eutorula sanguinea* forms an intensely light blood-red colouring matter Membrane is not slimy Assimilates the same sugars as the preceding species, and, like it, is destitute of fermentative power.

(*Torula*) *Eutorula rubefaciens* was isolated by Grosbüsch from apples The cells are oval-ellipsoidal It imparts a deep-red colour to must and mineral sugar solutions, and also to the layer of

gelatine under the colonies It produces an agreeable odour of fruit ethers, liquefies gelatine, and ferments saccharose, dextrose, and lævulose

The following two forms are distinguished from the preceding ones by forming jelly-like colonies on gelatine, and also by the fact that the plasma of the young cells does not contain oily corpuscules, the cells thus acquiring a different aspect. They form more acid, but less alcohol. Will retains for these two forms the name of Torula

Torula gelatinosa has spherical, sometimes tapering cells, under certain conditions much elongated. Membrane usually slimy The film is formed by flocks of slime uniting gradually. The deposit is slimy The nutrient liquid becomes turbid Beer-wort is coagulated and assumes the appearance of gelatine Giant colonies, brown, of a tough consistency Gelatine is liquefied. Ferments dextrose, lævulose, galactose, saccharose, maltose, lactose, raffinose, and arabinose Nutrient solutions are coloured yellow.

Torula cornicolor has spherical and ellipsoidal cells, in old cultures tapering Membrane stratified, the outside layer is apt to detach itself. Film slimy with ring formation The liquid remains clear Gelatine is liquefied. Giant colonies, dark brown, on wort-gelatine wax-like. Ferments the same sugars as preceding species. Nutrient solutions are coloured brown, also the rings, deposits and giant colonies In milk gives a cheesy smell

Second group Mycotorula is morphologically characterised by the fact that a branched or unbranched budding mycelium of elongated cells becomes more prominent These cells, however, never form a real mycelium The budding mycelium generates spherical or ellipsoidal cell forms, like conidia, resembling those of the first group, which, with cells of the same form, multiply through numerous generations by budding in series or in the same way as the saccharomycetes They are all aerophilous, liquefy gelatine and possess a higher fermentative power than the species of the first group They ferment dextrose, lævulose, galactose. saccharose, maltose, lactose, raffinose, and arabinose. Very inconsiderable, if any, formation of colouring matters. The upper limits of growth are at 35° and 40° C, respectively.

Mycotorula crateria, comprising several varieties, is characterised by the occurrence of *apiculatus*-like cells and the formation of crystals in the vacuoles Single-cell colonies on gelatine more or less sharply defined The name is derived from the peculiar structure of the giant colonies, old cultures on potato-water gelatine exhibiting in the well-marked varieties crater-like excrescences, followed by crested undulations one or more mm. in height.

Mycotorula radioplicata, likewise including a number of varieties, is remarkable for the radial folds that appear on the surface of

the giant colonies Among the cell-forms some resemble *S. apiculatus*, crystals form in the vacuoles.

Mycotorula turbidans belongs to another morphological series. It occurs in young beer, where it causes turbidity, which, however, disappears in the ripe beer It has elongated cells in budding associations, and short, rounded cells at the point of contact of two elongated ones It is more resistant to beer-yeast than the other species, and it does not require much air At the height of its development the cells clot together and then sink to the bottom. It does not ferment maltose, but it *does* ferment the other sugars named above

Between these and *Monilia*, Geiger observed a number of transition-forms, linked in chains partly of short cells and partly of very elongated cells, the latter branching off into cells of the same shape Geiger termed them *Pseudomonilia* and described several peculiar forms.

A species of *Torula* (*Torula Novæ Carlsbergiæ*), the cells of which exhibit very different forms, has been described by Grönlund It imparts a disagreeable bitter taste to wort According to Schjerning's investigations it inverts cane-sugar, and induces alcoholic fermentation in solutions of cane-sugar, dextrose, and maltose. In ordinary brewery-wort it can produce about 4·7 per cent. by volume of alcohol.

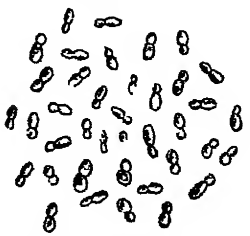

Fig 92.—*Torula a* Young culture (Brask) × 560

Another species, of special physiological interest, is *T. Wiesneri* described by Zikes and isolated from laurel leaves. It is able freely to assimilate atmospheric nitrogen In a pure glucose solution 2·4 mg N were combined for every mg of glucose assimilated, the amount being still higher on the surface of glucose-agar; after absorption of N, the dry matter in this case contained 3·1 per cent. of N (It may be noted for comparison that a normally nourished press-yeast contains about 4 per cent of N)

The following species of *Torula*, together with the two rose-coloured budding fungi described under the name of *Torula b* and *c*, were prepared in a pure state in the author's laboratory

J. C Holm gave an exact description of these three species.

Torula a = Torula Holmii n sp The culture of the young sedimentary growth, consists of small oval cells Here and there single larger cells occur both oval and round The length of the cells varies from 3 5 to 5 5 μ, the breadth from 1·4 to 2·1 μ. It gives a feeble fermentation in wort, yielding about 0·32 per cent. by weight of alcohol It inverts saccharose and raffinose, and ferments the inverted sugars It ferments dextrose, but not maltose, lactose, and dextrin (d puriss Kahlbaum) A film-

formation takes place in wort in three to five days at 25° C The cells of the film are round and oval in wort, whereas in dextrose-yeast water they assume *Pastorianus* or irregular forms The surface colonies on wort-gelatine (10 per cent) are round, white, lustrous, and slightly raised The edge of the colonies is smooth

Schionning submitted the *Torula* found by Claussen in *English beer* to a close examination, during which he isolated from different English beers some other species belonging to the same group He found that they fall naturally into two distinct groups Schionning selected a typical representative of each, described as *Torula* (*A*) and *Torula* (*B*)

Both develop slowly in ordinary lager-beer wort They give a typical low fermentation, which, in the case of *Torula* (*B*), lasts for six months at 20° C, whereas (*A*) ferments somewhat more rapidly

Torula (*A*)—The cells are elliptical, but at the same time sausage-shaped and even mycelial forms occur together with queer erratic shapes The size is somewhat variable Giant cells with strongly refractive protoplasm and thick walls are also found The protoplasm of the ordinary cells is feebly refractive, with indistinct vacuoles frequently containing a motile body

Torula (*B*)—The cells look somewhat like *Torula* (*A*), but are on the whole rather slimmer and more uniform, for the most part sausage-shaped, but long mycelial cells also occur In older cultures a loose layer of mycelium consisting of threads covers the true sedimentary yeast layer

In sterilised beer, which in the case of *Torula* (*B*) must be mixed with a little saccharose or glucose, slow propagation takes place at 25° C. The beer at first thickens, and afterwards gradually clarifies with the simultaneous formation of a somewhat coherent sediment Its cells are, on the whole, larger and more uniform, and the protoplasm is more refractive than in the corresponding wort-cultures If the development has taken place in closed vessels the beer gives evidence of a high content of carbon dioxide when it is poured out, and gives a fine head By storage in flasks with access of air both species form a feeble yeast ring and a film (like *Saccharomycetes*), the cells are elongated

Torula (*A*) may sometimes occur with a fine, dry, and greyish film like *Mycoderma*. The cells are then regular and elliptical

The temperature limits for propagation are—*Torula* (*A*) 40° to 40·5° C and 5° to 7° C, *Torula* (*B*) 39·5° and 3 4° C Optimum temperature 30° to 35° C for both groups It is worthy of note in this connection that the cells die in fifteen to eighteen months in wort, but if a little calcium carbonate is added life may be preserved for a long time, probably in consequence of the neutralisation of acid formed by the cells In bottled beer they can live for a long time

Saccharose is easily fermented by (*A*), and less rapidly by (*B*). Glucose and lævulose are easily fermented by both, maltose more readily by (*A*) than by (*B*) Neither ferments dextrin. Saccharose-yeast-water is fermented, but without giving Fehling's reaction, for although inversion takes place, the inverted sugar is immediately fermented Lactose is fermented only by *Torula* (*B*)

They are not very sensitive to the alcohol and acid formed during fermentation, so that at the end of the primary fermentation they can hold their own in competition with *Saccharomycetes*, and may, therefore, ferment the sugar residues with which the *Saccharomycetes* are incapable of reacting

When *Torula* (*A*) is added to fully-fermented beer (Danish export beer and Danish stout) or to wort, it forms acid along with alcohol, which to some extent combines with the alcohol and produces characteristic aromatic and flavouring ethereal substances The acid reaction of the liquid is also increased. *Torula* (*B*), on the other hand, cannot develop further in these beers, but by the addition of sugar a fresh fermentation sets in, with peculiar aroma and flavour This *Torula* is, therefore, unable to ferment the sugar residues if the beer has already been well fermented

In Danish, Swedish, and American beers these species can also be detected The conditions, however, are extremely unfavourable for their development, this is especially the case with the low temperatures used in the preparation of lager beer. According to Schionning, if pure cultures of these forms are introduced into Continental beers, they appear as true disease forms, imparting an unpleasant taste and smell to the beer.

Van Hest found a small (4 to 5 μ) oval or almost round *Torula* in top-fermentation Dutch beers. All these were opalescent or turbid, and possessed a peculiar fruity flavour. He termed the *Torula*, *Sacch pinophtorus melodus*, and found that under varying conditions its shape varies greatly, and that a mycelium is found in the film In wort it brings about a fairly strong fermentation. Another *Torula* (*S pinophtorus enervans*) is often found in the same beer. It is even smaller (2 to 6 μ) and round, produces less alcohol, and gives no aroma

Meissner found certain *Torulas* in old bottled wines and in viscous wines, which he termed "slime yeasts." He described eleven forms, some round, some oval, others large like *Pastorianus*. They do not ferment, but turn must, wine, and other liquids slimy The addition of ammonia greatly accelerates their growth, and brings about viscosity at an earlier stage. Larger quantities of alcohol hinder growth, this is also the case with the addition of sulphurous acid in the form of potassium bisulphite, even with the small quantity of ·05 per cent A similar effect is produced by the addition of 06 per cent of tannic acid. Feebly-fermenting

yeasts are at first suppressed by the slime yeasts, but at a later stage when the percentage of carbon dioxide increases these are themselves suppressed It is only wines poor in alcohol that turn viscous. A red wine which is rich in tannin is seldom affected

Hartmann cultivated in a pure state an organism derived from a dry yeast mass in Java, mainly consisting of rice starch named by him *Torula colliculosa* The size of the cells varies from 1·7 to 9·7 μ It forms a smooth and moist glistening surface on wort-agar, but within twelve to fourteen days numerous eruptions appear about the size of a pin's head In these are to be found the large cells This *Torula* ferments saccharose, glucose, raffinose, and lævulose

Adametz, in conjunction with Winckler, found two *Torulas* in Olmutzer Quargel cheese, one of which develops a yellowish-green fluorescent colouring matter on nutritive gelatine, and attacks the lactose, forming carbon dioxide but no alcohol. In kephir, Freudenreich found a *Torula* (*Sacch kefir*) along with three species of bacteria, which played a part in the fermentation It is small (3 to 5 μ) and oval, gives no fermentation in milk, but a peculiar yeasty taste When the milk has been hydrolysed, a process effected by the *Streptococcus* present in kephir, a fermentation takes place. Dextrose and maltose are both fermented

The yeast fungi which occur in strong salt solutions occupy a singular place Thus Wehmer has described a "salt yeast" which occurs in large numbers in pickled herring It is a small, round, or oval *Torula*, which thrives well in nutritive solutions containing from 10 to 15 per cent. of sodium chloride, and it remains capable of development for weeks and months in presence of 24 per cent. It is probably derived from sea water, and brings about, according the Wehmer, the formation of trimethylamine in pickled herring

In an examination of the ling organism (*Torula epizoa*), K. Hoye was led to undertake a series of air analyses along the coast of Norway. He utilised as a substratum a wheaten flour paste to which 17 per cent of sodium chloride had been added, in order to prevent the growth of the usual moulds and bacteria. On this salty substratum a few budding fungi developed, one of which gave spore-formation (described under the Saccharomycetes), the other two are *Torulas*.

Levure de Sel β is a roundish oval yeast, the shape of which depends on the amount of salt present By the addition of 15 per cent. of salt the cells are more oval than in the presence of smaller quantities, but with 20 per cent many are distinctly pointed, somewhat like a carrot Short fine points project from the membrane of the cells, two or three on each The cells are sometimes connected by these threads It does not develop in cider.

Levure de Sel γ is very variable in shape It grows like *Monilia*

in fish broth containing 15 per cent of salt It forms oval cells in chains in presence of 25 per cent, and gives absolutely round cells with 35 per cent of salt There is no growth in cider

According to Wehmer the sauerkraut fermentation is not simply a lactic acid, but is always accompanied by an alcoholic fermentation He states that three budding fungi occur with morphological differences, which he calls *Sacch. brassicæ I, II., III.*, but he adds that no spore-formation takes place, so that we are really dealing with *Torulas* The small species No 1 has an elongated spherical shape, No II is spherical, and No III., which is found most frequently, is ellipsoidal The yeasts are said to be the cause of foaming By destroying the sugar residues which have not been attacked by the lactic bacteria, they are of value in enhancing the keeping qualities of the preserved food According to R. Schulz, yeasts of the type of *S ellipsoideus* and *S. apiculatus* are present in the souring of beans, certainly in preserved raw beans Wehmer states that fermentation proceeds with the formation of gas

The red budding fungi (the "pink yeast" of medicinal bacteriology) were described by Will (see above). The following species belong to the same group

Torula b = *Torula mucilaginosa n sp.* The cells are oval and somewhat larger than those of *Torula a* (see above) with a length of 5 to 5·6 μ and breadth of about 2 μ.

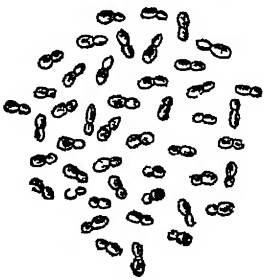

Fig 93—*Torula b*—Young culture (Brask) × 560

By sowing in wort a slight turbidity takes place at first, and almost immediately afterwards a slimy yeast ring forms on the side of the flask with a dirty red colour The ring increases in thickness, and grows towards the middle of the flask, so that this is gradually filled from top to bottom with a rose-coloured slimy mass. The clots of slime which fall from the ring form a more or less thick layer on the bottom On taking out the sample the liquid is quite ropy No fermentation takes place with dextrose, maltose, lactose, saccharose, raffinose, and dextrin Saccharose and raffinose are inverted By the cultivation of this species in wort with varying quantities of alcohol, a small yeast ring is observed in eight days at 25° C in wort containing 1 per cent. of alcohol. The ring is not formed in presence of 2 per cent., and no development whatever takes place in presence of 5 per cent of alcohol.

To determine how far the slime formation is influenced by the proportion of sugar and albumen, the following experiment was carried out In a pure dextrose solution the development was poor (1-5-10-20 per cent dextrose), and no yeast ring formed, only a minute sediment By the addition of peptone the ring

formation is restored When the quantity of sugar remains constant (10 per cent. dextrose), while that of peptone rises from 0·1 to 0·2, 0·5, and 1 per cent, the ring formation is favourably influenced With a constant quantity of peptone (0·5 per cent) and increasing quantities of dextrose (5-10-20 per cent) the slimy ring formation is reduced with increasing quantity of sugar. This shows that the slime formation depends upon the presence of albuminoids, and not upon that of sugar The surface colonies on wort-gelatine (10 per cent) are round, moist, and glistening, pale pink in colour, and slightly arched The young colonies have smooth edges, the older show a depression in the middle and slight transverse furrows at the edge

Torula c = *Torula cinnabarina n sp* The cells are predominantly of an elongated and oval shape, often provided with promycelium The length varies from 7 7 to 10·5 μ, breadth from 3·5 to 5·0 μ. Giant cells often occur, sometimes with rather elongated form, 14 6 μ in length, sometimes almost spherical and 9·5 μ in diameter

Fig. 94—*Torula c*—Young culture (Brask) × 560

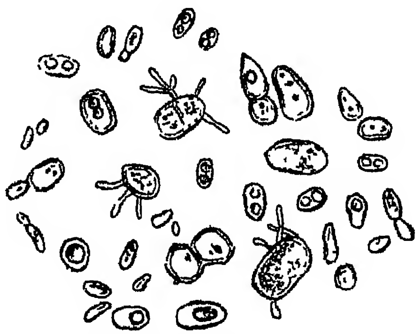

Fig 95—*Torula c*—Film-formation, old culture (Brask) × 560

When sown in wort or in various sugar solutions, it first forms a smooth and afterwards a dry wrinkled film with intense crimson-lake colour. The liquid under the film is clear No sedimentary yeast is formed, and no fermentation phenomena can be observed The wort undergoes a remarkable bleaching effect in older cultures. At 25° isolated islands of film appear on the surface in sixty hours, and few cells show indications of promycelium The formation of promycelium takes place freely in eighty hours The formation of buds takes place both on the promycelium and on the cells. There is no fermentation in dextrose, maltose, lactose, saccharose, raffinose, or dextrin, but saccharose and raffinose solutions are inverted

In wort with 1 to 2 per cent of alcohol a feeble fermentation is visible With higher percentages of alcohol, no development takes place The surface colonies on wort-gelatine (10 per cent.)

are round, pale pink in colour and opaque At a later stage the surface is warty, the edge jagged The old colonies are dry, and display a network of furrows and a finely fringed edge

Red *Torula* species occur in milk and cream, where they often form red specks on the surface, as well as in butter and cheese. Demme declares that the species occurring in milk and cheese is the cause of catarrh of the stomach in children

Kramer found a top-fermentation *Torula* in must which produces a red soluble colouring matter It ferments dextrose, and produces 4 5 per cent by volume of alcohol in a 10 per cent solution. Saccharose is inverted by this species, and maltose is directly fermented Lactose, on the other hand, is not affected

A considerable number of *Torulas* were found by Saito in the air at Dairen, Manchuria Only one of the nineteen growths described is capable of fermenting sugar (dextrose and saccharose); this species, *T fermentati*, has oval and elongated cells, and forms round colonies and a moist film, it liquefies gelatine. Temperature limits for budding, 35 5 to 40 5° C and 0·5 to 5° C.

Among other forms to be cited in this connection are:—*T. aurantiaca*, with orange-red colonies and globular oval cells, *T. sanguinea* with red, *T rufula* with light red, *T. corallina* and *T rubra* with deep red colonies—these five species containing invertase, *T minuta* with red colonies but without invertase; *T ramosa* with orange-coloured and *T. rubescens* with coral-hued colonies, both developing mycelium as well as the normal cell forms.

Torula Yeasts fermenting Lactose.

Duclaux found a yeast-fungus in milk which induces alcoholic fermentation in a solution of lactose This fungus appears to be most nearly related to the *Torula* species The cells are 1·5 to 2 5 μ in diameter, and almost spherical According to Duclaux's experiments, this yeast is more aerobic than the ordinary alcoholic yeasts. Even with strong aeration of the liquid, the whole of the lactose is used up in alcoholic fermentation. In a 5 per cent. solution of lactose 2·5 per cent. of alcohol was formed in eleven days at 25° C The most favourable temperature for the fermentation of a neutral solution is 25° to 32° C, whilst at 37° to 40° C the fermentation ceases Small quantities of acid have a retarding influence on the fermentative activity of this yeast.

Adametz likewise describes a budding-fungus which ferments lactose ("*Saccharomyces lactis*") Since this fungus does not yield endogenous spores, it must be classed in the group of non-*Saccharomycetes* The cells are of about the same size as those of brewery yeasts, and are spherical and elliptical. The colonies grown on peptone-gelatine are round, with slightly jagged borders, and are of a dark brown colour. A stab-culture in wort-gelatine

yields a dull, flat mass on the surface and a vigorous growth in the puncture channel, and from this numerous offshoots penetrate into the gelatine. In sterilised milk this fungus induces fermentation phenomena within twenty-four hours at 50° C, in forty-eight hours at 38° C., and in about four days at 25° C In this fermentation the lactose alone is decomposed

Both of the species mentioned above have been more closely investigated by Kayser, together with a new species, which likewise ferments lactose, and belongs to the *non-Saccharomycetes* All three yield colonies on gelatine, which are more widely spread than those of beer- and wine-yeasts, the colonies have a dense centre with a border resembling mycelium In milk and in neutral liquids, when sufficiently aerated, they induce an appreciable fermentation at 25° to 30° C The milk does not coagulate or become viscous during the alcoholic fermentation. All three species ferment lactose, galactose, cane-sugar, glucose, invert-sugar, and finally maltose, but the last only with great difficulty In the fermentation of milk-sugar with these yeasts, the resulting liquids are as rich in alcohol as the strongest beers Kayser remarks that it may, perhaps, be possible to make practical use of this observation and by means of these fungi convert the large quantities of whey, obtained in the manufacture of cheese, into an alcoholic liquor

Dombrowski found a number of *Torula* species to be of very frequent occurrence in milk and dairy produce (Many of them do not produce fermentation) He describes five different species, to which he gives the collective name of *T lactis* (α-ε) The first, α, isolated from Mazun—oval cells—is a top-fermentation type, no film, in milk, active fermentation with aromatic products Ferments lactose, dextrose, saccharose, and galactose, not maltose. Can yield about 5 per cent of alcohol Colonies in gelatine lenticular, β produces low fermentation, on solid media the cells are much elongated and mycelial, it ferments the same sugars as α γ from kephir with oval and globular cells, top-fermentation, film and ring-formation, colonies lens- and cone-shaped, reacts with sugars like α and β, δ and ε from butter have spherical cells (in δ considerably smaller) with large amounts of fat, ring-formation On gelatine same forms of colonies as the preceding species. None of the sugars named are fermented, but both forms will grow freely on yeast water with those sugars

Beijerinck has described two yeasts which also ferment lactose, and which must be provisionally regarded as *non-Saccharomycetes;* these are *Saccharomyces Kephyr*, which occurs in kephir-grains and consists of longish cells of varied shape, and forms slightly jagged colonies liquefying gelatine, and *Saccharomyces Tyrocola* (from Edam cheese), which consists of small roundish cells, and forms snow-white colonies on gelatine Beijerinck found that these two

species secrete a particular invertive ferment (*lactase*) which inverts lactose

In Lombardy Grana cheese a unilaterally budding, top-fermentation yeast was discovered by Bochiccio, which is called *Lactomyces inflans caseigrana* The growth consists of round, ellipsoidal, and oblong cells, and forms whitish colonies with smooth edges on gelatine No spore-formation was observed In lactose-broth it produces a vigorous fermentation at 25° to 40° C., the best temperature for development is about 30° C., the limit of existence at about 60° C Whey infected with this species is converted into a foaming beverage with a somewhat agreeable taste

Weigmann has isolated a pure culture of *Torula* from a defective butter. By fermentation in milk the products comprised 51·2 per cent. by weight of alcohol and 34·4 per cent of carbon dioxide, together with 3 6 per cent of butyric acid Orla Jensen has also isolated a *Torula* from butter which fermented maltose in addition to lactose P Mazé found ten different *Torulas* in soft cheese, one of which fermented lactose only, the others, in addition, fermented dextrose, lævulose, maltose, and saccharose. The fermentations are more rapidly carried to an end, and a higher yield of alcohol is obtained if they are carried out in an alkaline liquor Martin bouillon makes a good substratum with 0 088 per cent of sodium carbonate Mazé believes it to be probable that these species produce aromatic bodies in soft cheese. In American cheese and milk a *Torula* occurred at one time, producing a bitter taste. Harrison proved that the infection was derived from milk cans, which, in their turn, had been infected by exposure under maple trees to dry air The yeast-fungus, named by Harrison *Torula amara*, gives a strong and unpleasantly bitter taste to milk in fourteen hours at 37° C fermentation is brought about, and an odour developed resembling that of plum kernels, the flavour becomes more astringent At a later stage the milk curdles somewhat, and possesses a slightly acid and ethereal aroma. Lactose, glucose, and saccharose are easily fermented In milk the last trace of sugar is fermented The organism grows in broth containing 2 to 4 per cent of lactic acid

A complete and comprehensive description of these lactose-fermenting *Torulas* (together with the lactose-fermenting *Saccharomycetes*) is given by Heinze and Cohn They undertook a special and very detailed morphological and physiological investigation of Adametz' *Sacch lactis* and Beijerinck's *Tyrocola*.

Kalanthar detected three lactose-fermenting species in Mazun —among them the genuine Mazun yeast with giant colonies, which are first greenish-grey and then plum-red It ferments lactose, saccharose, trehalose, dextrose (feebly), but neither maltose nor a-methyl-glucoside, it also produces acid.

In many defective butters and cheeses, *Torulas* other than the

above have been detected, which appear to be of more or less importance, thus Roger found a *Torula* in a fishy and rancid butter, which occurred several times in cases of preserved butter, and contains a fat-cleaving enzyme Adametz has observed a *Torula* during the blistering of cheese, which was also discovered by Bochiccio in Lombardy Grana cheese The *Lactomyces inflans caseigrana* alluded to above brings about a marked blistering on the outer parts of hard cheese It coagulates sterile milk, and partially liquefies the coagulum without noticeable formation of acid It must, therefore, contain a clotting enzyme and a tryptic enzyme

Saccharomyces apiculatus Reess.

This fungus was discovered by Reess (1870) in fermenting fruit-juices and in wine-must, and also as a regular constituent of the fungoid flora on ripe grapes and tree-fruits. He showed that it appears in different forms in the course of its development, the typical one being the lemon shaped, he therefore gave it the appropriate name of *S apiculatus*

This ferment was the subject of one of the finest and most thorough biological investigations of our time, for Hansen was enabled, after several years' work, to determine both its habitat in nature and its regular migrations at different seasons of the year.* The reason why this species was selected for the investigation was that it could always be recognised in cultures by its lemon-shaped cells

S apiculatus, besides occurring in wine fermentation, is also found in spontaneously-fermented Belgian beer (Lambic, Faro, Mars, Krieckenbier), and, according to van Laer, imparts to it its peculiar taste and odour

If a little of such a growth is examined under the microscope in a drop of nutritive liquid, the development of the fungus can be followed This is very characteristic (compare Fig 96) It is seen that the buds formed from the typical lemon-shaped cells may be either lemon-shaped (*a*, *b*, *c*, *e*, *f*) or oval (*a-c*), it will also be noticed that the oval cells must first form one or more buds before they are able to assume the lemon-shape (*e-f*), and finally, that the lemon-shape of a cell attained by budding (*k*, *k'*, *k''*) may be lost again on the formation of a new bud (*k'''*). Under other conditions the cells may assume quite different forms, sausage-shaped, crescent-shaped, like bacteria, etc. (*g-m*) Does any rule govern this apparent confusion? It was shown by means of culture experiments that the lemon-shaped buds are developed especially during the early stages of the culture, but are afterwards crowded out by the oval forms.

* See section: Biological relationship of yeast

The species examined by Hansen will now be further described from the physiological and biological standpoint

Sacch apiculatus is a bottom-fermentation yeast, capable of exciting alcoholic fermentation in beer-wort, the fermentation in this liquid is, however, a feeble one, only 1 per cent by volume of alcohol being produced, whilst a bottom-yeast under the same conditions gives 6 per cent This arises from the fact that the Hansen species cannot ferment maltose Hansen also found that this species does not secrete invertase On the other hand, it excites a vigorous fermentation in 15 per cent and 10 per cent solutions of dextrose in yeast-water, and in one experiment as much as 3 per cent by volume of alcohol was formed After three

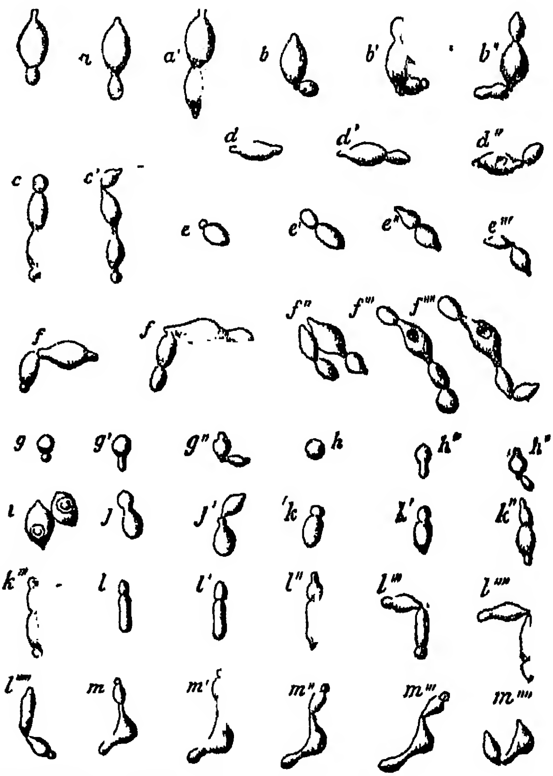

Fig 96—*Saccharomyces apiculatus* (after Hansen)—Budding cells *a-a''*, a cell which in the course of 3¼ hours developed a bud at its lower extremity, *b-b''*, a similar series, showing the development of a bud at the upper extremity of the mother-cell, whilst a bud had been previously formed at the opposite end, *c* is a chain of cells. *c'* is the same three-quarters of an hour later, the lowest bud had assumed the typical form of the species like those above it, but in the figure it is seen from the end, so that its longitudinal axis is at right angles to the plane of the paper, *d-d''*, development during 1¼ hours, *e-e'''* during 2¼ hours; *f-f''''*, during 3 hours, in *e-f* it is seen that the oval cells first develop a bud and only subsequently assume the typical lemon-shape, *g-m*, abnormal cells, progressive development.

months the liquid still gave the sugar reaction whilst the amount of alcohol had not increased during the last six weeks The fungus was thus unable to complete the fermentation. In another of Hansen's experiments as much as 4·3 per cent by volume of alcohol was produced. Also lævulose and mannose are fermented

The temperature limits for budding in wort were found by Klocker to lie between 36°-37° C and 3 5°-0 5° C It survives an hour's exposure in wort at 45° C, but not at 48° C At 35° C. in wort the cells after three days become swollen, and many of them take the form of long sausages

The organism is very sensitive to chemical reagents Sulphurous acid (0·025 per cent) almost entirely prevents its fermentative activity, and alcohol acts very restrictively. On the other hand, tannin only acts at a strength of 0·5 per cent

It was found by experiment that this fungus, being the weaker species, was crowded out from a mixture with *Saccharomyces cerevisiæ* grown in beer-wort, although it retarded the growth of *Sacch. cerevisiæ* to no small degree

In flasks with the same beer-wort, and at the same temperature, each containing one species, *Saccharomyces apiculatus* will multiply to a greater extent than the brewery yeasts in a given time.

At the critical time of the year, the ferment, if present in the wort in considerable quantities, may exist for a length of time side by side with brewery yeast, and will no doubt retard its action a little; but when the beer is transferred to the lager cellar, the fungus remains inactive in the alcoholic liquid, and frequently perishes.

Muller-Thurgau and Wortmann regard the fungus as injurious to wine, for it not only directly prejudices the quality of the wine and must, but also checks fermentation, and thus gives rise to disease.

The organic non-volatile acids (tartaric and malic) present in fruit juices and grape must are attacked by *S apiculatus*. It is possible that they serve as sources of carbon for this yeast, or else that they are decomposed in the fermentation process As fast as the acid is consumed a fresh formation of acid, especially volatile acids, takes place

Hansen, as already stated, always made a single species the object of a research. It was afterwards proved that the specific name embraces a number of species or varieties characterised by the typical lemon-shape of the cells, which predominates, not only in the first stage of development of the cultures, but also later, under suitable nutritive conditions Many of these species occupy a peculiar position in the budding process also, when the daughter-cell separates from the mother-cell, a new bud appears at the point where the separation took place The morphological differences between the species or varieties are less considerable Spore-

formation is only known as yet in very few species Beijerinck met with some spores in his cultures (1894), he does not state, however, whether they could germinate Later, in cells from flowers of a Robinia, Lindner observed a single spore with a distinct wall and granulated plasma but no germination, after cultivation in wort in a moist chamber Rohling found that several cultures formed spores on gypsum blocks, and succeeded in observing germination in an extract of horse-dung with 5 per cent dextrose Similar observations were made by Holm in the author's laboratory working with lemon-shaped cells, somewhat larger than the ordinary species, but without germination Klocker, on the contrary, could observe in one species a typical abundant sporulation and successive phases of germination, he, therefore, gave this species a special generic name, separating it from all other species, where no spore-formation has as yet been observed

The principal distinctive marks of the varieties are physiological

Thus, Amthor discovered considerable differences between the races in the amount of volatile acids or alcohol and glycerine produced Marked differences were also found by Müller-Thurgau between the races he experimented upon, some of which produced only 2·5 per cent of alcohol by weight, while in others the amount was considerably higher, rising in one case to 6 per cent. Rohling also established several physiological facts in the course of his work

Very extensive physiological investigations have been carried out in recent years by Klöcker and Will

The former, in his studies of seventeen different forms, proved the great influence exerted by temperature on the shape and dimensions of the cells

All these forms, except one, are classed by him—because no spore-formation has been observed in them—among the Torulaceæ, as a subdivision, which he calls *Pseudosaccharomyces*. They were tested with dextrose, lævulose, saccharose, maltose, *d*-mannose, and do not ferment lactose or galactose They are classified as follows :—

The *first group*, to which belong the Hansen species already described, does *not* contain *invertase* Two of the species included in this group ferment small quantities of maltose. The others are distinguished by their yield of alcohol, minimum temperature of budding, etc

The species of the *second group*, originating from tropical countries, contain *invertase* and ferment invert sugar In this group there are marked differences in regard to alcohol production, limiting temperatures of budding, nature of sedimentary yeast, cell-forms at high temperature (35° C), and so forth.

A peculiar position is occupied by the seventeenth species,

which forms *spores*, and is termed *Hanseniospora Valbyensis* Like the other species, it has not only ellipsoidal and elongated, but also lemon shaped cells. The temperature limits for budding in wort are 32° 33° C and below 0·5° C respectively It does not withstand being heated one hour at 45° C The young spores are globular, later they become semispherical, surrounded by a rim at the base There are usually two spores in a cell. It ferments dextrose, levulose, and *d* mannose, and produces up to about 7 per cent. of alcohol. After one month in wort the percentage was 0·93.

Will thoroughly studied four non-sporulating species, two from recently fermented beer-wort and two from grapes. In accordance with Klocker, he classes them among *Torulaceæ*, which comprise some species with lemon-shaped cells, though these are far from typical or predominating. Will gives a full description of the budding process and the cell-forms, including the typically lemon shaped giant cells, in all the species examined by him he found that important characters for determining the species are given by the cell forms in the second stage of development, the film-formation. Long cells appear in two species at this stage, while in the other two they are round or slightly elongated. He further shows that the structure of the giant colonies is very peculiar and constitutes a *particular type*, differing from that of the colonies formed by other budding fungi The upper temperature limit of budding was between 34° and 35° C. It was not possible, however, by means of the characters studied and compared with the description of Klöcker's species, to identify the four species with any of the latter.

Mycoderma.

It is characteristic of this group that it very readily forms films on various alcoholic liquids. Under the name are included a number of different species, some of which may excite a feeble alcoholic fermentation; they behave differently towards lager beer, some causing disease whilst others do not. In wine they are dangerous disease germs.

The *Mycoderma cerevisiæ* examined by Hansen, which is universally met with in Copenhagen breweries, forms variously-shaped cells. They are usually transparent and less refractive than the true *Saccharomycetes;* in each cell there are generally one, two, or three highly refractive particles, which often have a quivering, rolling motion. This micro-organism forms a dull, greyish, wrinkled film on wort and beer, and does not excite alcoholic fermentation; neither does it invert solutions of cane-sugar.

The colonies on the surface of the gelatine are light grey, dull, and spread out like a film or hollowed like a shell. By means of

this macroscopic appearance *Mycoderma* is readily distinguished from the ordinary *Saccharomycetes*, which, on the same medium form light greyish-yellow colonies with a dry or lustrous surface and a more or less arched form

The film-formation was noted by Hansen when lager beer had been exposed in open vessels at temperatures between 2° and 15° C, at 33° C development still occurred, but at temperatures above 15° C this species gave place more and more to competing forms. As low temperatures are favourable to its development, it will readily thrive in the storage cellar, especially as lager beer forms a much more favourable medium for its growth than wort This is seen to be the case when traces of a pure film are introduced into lager beer and wort, contained in open vessels, and then left to develop, the culture in lager beer nearly always remains pure, while in wort various other species make their appearance

In Hansen's comprehensive experiments on Carlsberg beer, it was always found that both lager and export beers were attacked by this fungus, but there was never the slightest indication that the beer had acquired any disease from this source The fungus was widely distributed just at those periods when the beer was found to be particularly stable and of good flavour. This has also been confirmed by numerous experiments on lager and export beers carried out in the author's laboratory It is self-evident that we are only speaking of beer which has been properly treated In imperfectly closed bottles and casks, *Mycoderma cerevisiæ* will, of course, rapidly develop a film, which is sufficient, unaided, to destroy the product

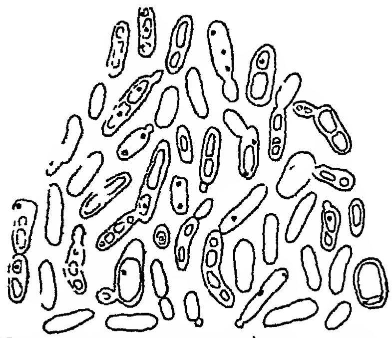

Fig 97—*Mycoderma cerevisiæ* from Copenhagen breweries (drawn from nature by Holm)

In yeast-making, more particularly in the manufacture of air-grown yeast, several *Mycoderma*-species come into play, because they meet with favourable conditions of development. In the froth formed by the stream of air may often be found a considerable infection of *Mycoderma* cells, generally derived from impure pitching yeast and favoured in their growth by insufficient acidification of the fermenting wort

Hansen expressed the opinion that the name *Mycoderma cerevisiæ* denotes not one, but several different species, and Lasché's experiments subsequently confirmed this. The latter investigator describes four different species which he isolated from cloudy beers. They are distinguished from the species described by Hansen

by the fact that they produce alcohol in beer-wort, one yields 0·26 per cent by volume, two yield 0 79 per cent, and the fourth 2·51 per cent Lasché concludes from his experiments that these four species cause diseases in beer, both turbidity and changes in taste and odour, in this respect they also differ from Hansen's *Mycoderma* Lasché is inclined to assume that the chemical composition of the wort has no influence on the disease caused by *Mycoderma*, for, in his experiments, the disease was produced in worts of high extract and worts of low extract, in worts rich in sugar and worts poor in sugar.

Winogradsky found that the *Mycoderma* occurring in wine, prepared in pure culture by Hansen's method, alters its shape with the composition of the nutritive solution, he experimented both with solutions, the mineral constituents of which remained constant while the organic substances varied, and also with solutions in which the reverse was the case

Many experimenters have subjected *Mycoderma* to close investigation during the last few years, and especially Henneberg, Heinze, Meissner, Seifert, Will, and Dombrowski Meissner's researches are particularly comprehensive, both morphologically and physiologically, and they concern twenty-three different species of *Mycoderma vini* Greater or less distinctions were noted in the shape of cells, glycogen content, presence of oil drops, and giant colonies The film-formation also showed distinct features in different species, both with regard to the time required for its appearance, its character, and its colour (white, cream, yellowish-brown, and yellowish-olive-green) When covered with the film, the liquor remains clear in certain cases, whilst in others a turbidity, of a permanent or temporary character takes place

Will believed that the decolorisation of the liquid may be due to the formation of acid brought about by film cells, whereas Heinze thought that it was caused by the removal of acid Meissner confirmed the fact that a more or less marked decolorisation takes place, but showed that afterwards a reversal of the colour tints may come about, so that must which had turned pale assumed by degrees a dark brown colour Not only is the total amount of acid destroyed, but the must at last acquires an alkaline reaction. Meissner succeeded in proving experimentally both with large and small amounts of must, that one and the same race may appear, first as a producer, and then as a destroyer of acid. It is, therefore, necessary to forego the division of film yeasts into acid-destroying and acid-forming species Once the sugar in the must is destroyed, all so-called acid ferments will act as destroyers of acid With regard to this question of the formation and destruction of acid, Meissner also states that when an increase of acid takes place it must be regarded as a result of two simultaneous processes of construction and destruction, and that

production has exceeded destruction If, on the other hand, a reduction of acid takes place, the destructive action must be regarded as exceeding the constructive

Butyric acid is formed amongst others, and ammonium compounds are also produced

Meissner's experiments regarding the reaction of the *Mycodermas* with organic acids gave the following results:—Malic acid is only very slightly attacked by certain races, but strongly attacked by others Tartaric acid is slightly decomposed Lactic, citric, and succinic acids are in some cases strongly affected Acetic acid is strongly attacked by a few species; in other cases the species cannot grow at all in a solution containing acetic acid.

Alcohol, the sugars, glycerine, and tannic acid are decomposed. Alcohol is converted by an oxidation process into carbon dioxide and water, but may also act as an organic foodstuff As early as 1878 Schulz found that "the film fungus can produce within itself ready formed organic compounds, and requires nothing but ammonia and alcohol for the purpose" Schulz did not, however, work with pure cultures

Meissner utilised for his experiments both the nitrogenous nutritive material used by Schulz (ammonium nitrate, asparagine, ammonium tartrate) and an artificial solution containing ammonium phosphate and ammonium chloride, together with the necessary mineral constituents The vigorous growth of *Mycoderma* proves that these two solutions supply them with nitrogen. With regard to the sugars, Meissner found that the *Mycoderma* species cultivated on sterile grape juice respired dextrose and lævulose to some extent, partially producing acids from them On artificial nutritive solutions which contain dextrose or saccharose as the only organic substance in addition to the necessary mineral nutrients, the sugars are oxidised, part being utilised for the construction of new cells, and part for the fresh formation of acid. Glycerine is not only destroyed, but may be produced from other organic substances This fact was confirmed by W Seifert.

Wortmann has drawn attention to the fact that many wines which have become filmy have an odour which resembles rancid butter to an extraordinary degree Butyric acid has been formed in these cases

Lafar found in a cask store a *Mycoderma* which imparted a flavour to beer resembling wine ether

In finished wines the yeast has generally, but not always, finished its special work, but nevertheless wine may undergo fundamental alterations caused by other micro-organisms, and amongst them, according to Wortmann, *Mycodermas* take the first place By their agency, alcohol is converted into carbon dioxide and water, and they also influence the amount of acid and destroy the bouquet A wine may become filmy, and may

deteriorate in time without a visible covering of film appearing on its surface. The suspended cells of *Mycoderma*, which need not be numerous, may bring about in the course of years the same action which would occur more rapidly if the cells formed a coherent mass on the surface. *Mycodermas* which often inhabit corks (like the moulds) may impart the well-known corked taste to wine.

Will has isolated a *Mycoderma decolorans* from top-fermentation beer, which brings about a marked decolorisation of beer. This occurs within a short time at the high temperatures requisite for completing top-fermentation. The species develops a large amount of acid in beer, but under certain conditions a destruction of acid may also take place. It cannot bring about alcoholic fermentation. Will also made a series of observations on the duration of life in wort-cultures and in the dry state, and on the power of resistance to heat in liquids.

Will and Leberle further described four other species, which were grown on yeast water and dextrose. They remark that the chief specific characters were brought out by physiological experiments. One of these species, *M. cerevisiæ a*, is distinguished by its inability to assimilate dextrose. Lævulose is assimilated by all four species, while they are inactive to saccharose, maltose, lactose, and galactose. Alcohol proved an excellent nutriment. They vary in their power of assimilating organic acids.

Seifert closely examined two *Mycoderma* species isolated from wine, which produced from 0·064 to 0·904 per cent. of acetic acid in an ordinary Austrian white wine, and reduced the amount of alcohol.

Four types occurring in wine were described by Rossi as distinguished by the shape and dimensions of cells, temperature limits of growth and power of resistance to alcohol. None of them produced fermentation in must.

A species which seems to be classified with this group, *Pseudomycoderma vini*, was found by Will on grapes. The cells are small, spherical or ellipsoidal, others are spindle-shaped or resemble *S. apiculatus*; they are often elongated and form large budding associations. The long forms have an oily corpuscle in the plasma and crystals in the vacuoles. It almost always forms films, beginning, on wort, as islets looking like solidified oil drops; at a later stage the film is surrounded by a thick, raised edge and wrinkles, taking on a yellowish-brown or reddish colour. Colonies on gelatine are very irregular, with strong radiations of mycelial cells. Giant colonies wrinkled, with knots. Gelatine is liquefied. Dextrose and lævulose are actively fermented. Formation of colouring matter very scarce.

The *Mycoderma* investigated by Heinze (*M. cucumerina*, Aderhold) was derived from a fermentation of sour cucumbers. he

declares it to be a dangerous enemy of lactic acid fermentation. In beer the organism attacks alcohol strongly, and produces a bitter flavour It is capable of producing alcoholic fermentation in dextrose solutions In cider, with 10 62 g of sugar, it yielded 4 34 g of alcohol per 100 c c of fermented liquid, in five months, at 25° C There is no fermentation with maltose, saccharose, and lactose

Henneberg mentions two species of *Mycoderma*, which he frequently found in distillery and pressed yeast The shape of the cells is very varied The one species frequently forms mycelial, *Monilia*-like chains The difference between the two forms is specially marked in cultures on solid substrata In dextrose and lævulose solutions bubbles of carbon dioxide form under the film, produced by the cells, which sink to the bottom Both species produce acetic ether The optimum temperature for growth lies between 32° and 41° C Dextrose and lævulose are readily fermented, galactose less readily, only traces of maltose and dextrin are fermented, whilst lactose, saccharose, raffinose, and inulin are not fermented at all In dextrose solution about 4 per cent. by volume of alcohol is produced The two species can readily utilise lactic acid as food, and withstand up to 5 per cent of the acid. Similarly they can withstand large quantities of alcohol (11 per cent.) The alcohol in this case is fairly quickly converted into carbon dioxide and water They are very sensitive to acetic acid ; at 0·75 per cent growth is fully arrested

Mycoderma lactis was isolated by Dombrowsky from butter and rennet The cells are rectangular with rounded edges , some are elongated, sickle-shaped, or spherical It soon forms a film and deposit Colonies on gelatine, flat with an edge resembling mould. It ferments dextrose with formation of ester In 100 c c of grape-must up to 6 g. of alcohol are formed.

Mycoderma Chevalieri, found by Guilliermond in a kind of ginger-beer (Africa), produces a weak fermentation in wort and ferments saccharose, dextrose, and, actively, lævulose and *d*-mannose On wort it forms a greyish-yellow film, which soon sinks to the bottom and is replaced by a new film Cells oval, more or less elongated , in old bottom growths mycelial. On wort-gelatine at 20° C. the colonies are yellowish-grey with a dry surface The large colonies have a yellowish reticular central part, surrounded by a white frame with radiating stripes.

Saito described several other types (Japan), one of which, isolated from soy-beans, offers particular interest on account of the very irregular forms sometimes assumed by its cells The colonies on gelatine are greyish-white with a moist surface, the centre of which is slightly elevated, and the borders crenelated. It does not liquefy gelatine The large colonies have a dry, mealy surface. On a decoction of koji it forms a white, dry film with

large intervals, which afterwards become pleated and mealy. It does not ferment the common sugars

In Egyptian Leben (Leben raib), Rist and Khoury found a *Mycoderma* about which they say that it is not improbable it has a particular influence on the special flavour of the Leben ; in any case the rapid development of a sharp acid taste, which renders the beverage undrinkable in a few days, must be ascribed to this organism It forms both non-volatile acids and acetic acid It grows excellently in glucose and maltose, and gives a fermentation with the former, whilst it converts glucose into acid, and brings about the combustion of alcohol In lactose solution it gives no fermentation, only film-formation

CHAPTER VI

THE PURE CULTURE OF YEAST ON A LARGE SCALE.

Industrial Application.

By the industrial application of pure cultures of systematically selected yeasts inaugurated by Hansen and the author, it became possible to carry out fermentations with certainty in a way that was impossible so long as an unknown yeast-mass was used containing not only a mixture of culture yeasts, but also wild yeasts, bacteria, and even, in certain cases, moulds Such selected races can be preserved by appropriate means for a long time as small cultures which can be developed afresh into mass cultures.

One very important result of the adoption of the process was to prove that the visible fermentation phenomena do not in general give any clue to the purity of the fermentation On the other hand, these phenomena may sometimes give valuable information regarding the condition of the yeast, which is directly connected with the nature of the nutritive liquid.

A real knowledge of the purity of fermentation can only be gained by a biological analysis combined with a microscopical examination.

Pasteur demonstrated the harm that bacteria can do when they develop in alcoholic fermentation, and at the same time he emphasised the importance of the oxidation of the nutritive liquid for yeast activity. Hansen experimentally proved that some of the most dangerous diseases of beer are caused by wild yeasts *

Before the relatively small quantity of *pure yeast grown in the flasks* can be utilised on an industrial scale, it has to be further developed in the factory, where it must be introduced until the required quantity has been secured Owing to the great power of adaptation possessed by alcoholic yeast, it is always possible

* The "natural" cultivation of yeasts proposed by Delbruck must not be confounded with the preparation of a single pure race His process consists in subjecting the whole impure yeast-mass to a treatment which may consist of the application of a higher fermentation temperature, or pumping into a new vat after the appearance of foam on the surface, or pitching with wort from the first stages of fermentation, etc A summary process of this kind will always yield an uncertain result, because the impure mass contains elements of very different character, and even in the most favourable case, if by good luck the disease germs are restricted, it is evidently impossible to depend on securing the best-selected type of culture yeast It is essential to isolate the different yeast species and then to select those which best fulfil the stated requirements

that, during the long course of development it has to undergo—in the moist chamber, in successive flasks, and during the small-scale preliminary fermentations in the factory—some one species may undergo variation regarding the very properties that are of value in practice. In the preliminary fermentations the main point is, therefore, to endeavour to approach as near as possible to the conditions obtaining in the particular factory, with a view to maintaining, as far as possible, the normal character of the yeast employed. At the same time care must be taken that any infections to which these conditions may give rise are prevented by suitable means so that a practically pure yeast-mass for the large scale work may be secured. This has proved possible by making a proper choice of nutrient liquid, temperature, and quantity of liquid. Obviously the nutritive liquid should be essentially the same as that usually employed in the factory, and the relation between the quantity of yeast added and the temperature at which it is to work should be such as to insure a quick appearance of fermentation phenomena in the small fermenting vessels. The whole of the fermenting liquid should then be run at once into a larger volume of liquid, and this operation should be repeated until fermentation has been induced in one of the large tuns.

The proceeding here described has been found to be suitable in the great majority of cases, the yeast mass having exhibited its essential normal properties.

The dimensions of the small fermenting vessels are generally such that the diameter of the surface of the liquid is equal to about one half of the height: they are provided with a loosely fitting lid. If they cannot be placed in a room having about the requisite temperature for small scale fermentation, they must be covered with insulating material, or placed about 18 inches above the floor level and heated gently.

Brewers' bottom fermentation yeast may be dealt with as follows —

The yeast, after being developed in three or four Carlsberg cans, is run into 5 litres of wort of the usual kind, cooled to 19° C. In the course of a few hours generations of new yeast-cells will be formed, and the liquid is then run into a small vessel containing 50 litres of wort at 16° C.; when it is covered by abundant foam, the liquid together with the yeast deposit, is run into a larger vessel of 6 hectolitres capacity. The first development of the 55 litres is effected in one hectolitre of wort at 16° C.; after the foam has formed, $3\frac{1}{2}$ hectolitres at 12°-15° are added. The resulting 5 hectolitres are allowed to undergo an active fermentation and then, together with the yeast deposit, are run into about 40 hectolitres in an ordinary tun at about 10° C. When abundant foam has covered the surface, the tun is filled up and the fermentation completed. If the yeast culture has travelled a long way—whether in liquid or dry form—the cells are first rejuvenated at about 25° C., and the

first fermentations are carried out at temperatures a few degrees higher than those quoted above

With *Brewers' top-fermentation yeast* the same procedure is followed, the temperatures being regulated in accordance with those adopted in the large-scale fermentations The quantities of wort employed in the successive fermentations may be somewhat larger as a rule. Only in the case of a few particularly sensitive top-fermentation yeasts has it been found suitable to complete each small fermentation and to use the "mature" yeast obtained for pitching purposes.

In the first large tun the fermentation, whether bottom or top, will in general show some deviation from the normal course, but not more than can be readily corrected in the secondary fermentation in the casks, where the beer is delivered from a number of tuns

In *Distilleries* the acclimatisation of pure yeast can be effected with greater surety, as it is developed under the same conditions as in the large-scale fermentations The culture is introduced into a small quantity (5 to 10 litres) of the common concentrated yeast-mash acidified by a lactic ferment, and after it has set up a vigorous fermentation, this small portion of the mash is introduced into about ten times the volume and the process repeated Only the first portion is boiled, the subsequent fermentations are conducted in ordinary mash. The first propagation should take place at 25° C, the next at 23° C In either of these fermentations, care must be taken to prevent the temperature rising more than two or three degrees (regulating either by water-bath or room-temperature), the following fermentation is induced at 18° C and carried out at a temperature rising gradually to about 27° C

A similar procedure is adopted in the propagation of pure yeast in the *Vienna* system of fermentation.

In *Molasses* fermentation, on account of this material being less readily fermentable, it has been found beneficial to add, to the first small portions of molasses (12°-10° Ball), a slight dose of malt-culms or yeast-extract, and afterwards to boil the liquor and acidify it with sulphuric acid to about 0·7 N The fermentation is carried out at 23°-25° C, the first portion of molasses does not exceed 20 litres, a similar quantity being added each time when about one-half is fermented In later stages the degree of dilution of the molasses must be the same as in the large-scale fermentations. If the presence of too many bacteria in the molasses necessitates an addition of bactericidal substances, such as hydrochloric acid and formol, the pure culture must be acclimatised, in the laboratory where it is prepared, to the particular composition of the medium in which it has to work

As for *Air*-grown yeast manufacture, several special races of yeast are in use In propagating the pure culture the yeast must be prepared to withstand the full current of air which is necessary

to make the yeast-cells multiply freely The following is the simplest procedure: The culture is first made to work in 25 to 30 litres of original wort which has previously been boiled for half an hour and cooled to 26° C.; in the course of fermentation the temperature is allowed to rise to 30° C. When in a state of vigorous development, the fermenting liquid is run into some 300 litres of unboiled, original wort at 25° C., during fermentation the temperature rises to 29° C. The fermenting liquid is then run into 1,600 litres of wort of about 6 per cent. Ball at 24° C, with a slight supply of air, the temperature rising to 28° C. The yeast yielded is used for pitching, with due regard to the working methods of the factory

In *Wine* fermentation the application of selected yeast races is being adopted, Müller Thurgau and Wortmann having demonstrated that these yeasts range themselves in typically different groups (Champagne, red wine, Rhine wine, and other types), which retain their characteristics under varying conditions.

The difficulty of making any selected race assert itself is due to the well known fact that wine must contains numerous living germs of very different kinds, which are apt to enter at once into competition with the pure yeast; the assumption being that, after a normal fermentation, the sediment will contain the predominating good type, surviving the secondary fermentation.

As for the method of using the pure yeast grown in the flasks, it may first be propagated in 10 to 20 litres of sterilised must. The product may be employed direct as seeding yeast, or, when large quantities of grapes are used, it may serve (in a vessel fitted with a bung-hole and tapping-cork) to prepare a larger amount of seeding yeast, with the addition of 1 hectolitre of fresh must, not yet fermenting and not boiled up. From 0·1 to 2 per cent. of the fermenting culture is used for seeding the must (the yeast sediment being stirred up), according as the must is more or less rich in sugar; some regard also being paid to the temperature and the biological condition of the must. The amount required should bring about a quick, but not violent, fermentation, the latter being apt to hinder the formation of bouquet substances. To prevent the germs of the must from developing, it is advisable to pitch the yeast at as early a stage as possible. The prepared culture may be added to advantage, in many cases, particularly in a warm autumn, to the fresh must in the vineyard.

Among the various ways in which trials have been made to further the work of the selected yeast-race, the most practical is doubtless the addition of disinfectants to the must, especially since Müller-Thurgau has shown wine yeasts to be in general more resistant to sulphurous acid than are the other ferments in must. Obviously must cannot be absolutely sterilised by this acid or by potassium metasulphite ($K_2S_2O_5$), as considerations of flavour, etc., make it necessary to keep within narrow limits. As a matter

of fact, considerable advantages are derived from thus weakening any competing germs, providing that the pure culture is acclimatised to the added chemicals by suitable treatment, so that it is able to develop with full vigour

According to Muller-Thurgau, an addition of 40 to 120 mg of sulphur dioxide per litre is sufficient, the exact amount being determined in accordance with the composition of the must and the conditions under which the fermentation is carried on. Other workers used up to 200 mg , 20 g of potassium metasulphite per hectolitre have been successfully used with pure cultures adapted to sulphurous acid. Part of these antiseptics in solution may be poured over the grapes before mashing

One way in which a pure yeast culture can be adapted to sulphurous acid is by refreshing it in 2 or 3 litres of sterilised must, and then adding to the vigorously fermenting must 100 c c of must treated with 50 mg of sulphurous acid , after fermentation has set in, similar doses are added in succession until a volume of $2\frac{1}{2}$ litres is obtained, containing about 0 01 per cent of sulphurous acid. This yeast-mash is used to set up fermentation in a larger quantity of must with the same content of acid, at 18°-20° C , and the resulting liquor is distributed among the casks or tuns

When the fermentation of wine comes to a standstill for some reason or other, the pure culture should be first developed in about 20 litres of the wine, which, after adding some 2 kilos. of sugar, is boiled for twenty minutes and cooled to 20° C The fermenting wine may be added to 12 hectolitres of wine that has just received a suitable dose of sugar When fermentation has set in, the whole batch of wine is used to ferment correspondingly larger quantities of sugared wine

To re-ferment wines that do not contain a sufficient amount of alcohol, or that are too acid, it is advisable to add 1 or 2 litres of liquid pure culture per hectolitre, according to the amount of alcohol present The wine, drawn off from the old yeast, must have been dosed with sugar just before pitching

As regards after-fermentation of old wines, Wortmann recommends adding sweet must in sufficient quantity to give 1 or 2 g. of sugar per litre, and 1 c.c of thick-liquid yeast per litre

It will be understood that these indications must be adapted to the conditions obtaining in each particular case

In *Fruit-wine* fermentation the application of pure yeast is of very great importance on account of the many different epiphytes contained in such juices It is, therefore, necessary to pasteurise the juice or to have recourse to the chemicals referred to

This kind of fermentation is usually set up by selected wine yeasts , according to Müller-Thurgau, however, the yeasts occurring in apple or pear juice may also serve the purpose if judiciously selected Considerably more seeding yeast is required to set up a

quick and vigorous fermentation than in the case of grape-wine fermentation.

The use of pure cultures is of particular importance in fermentation of sparkling wine, where it is possible, by submitting the selected race to a suitable treatment during its development, to make the yeast settle quickly to the bottom and to form a granular sediment. Comparatively small quantities of yeast are required, about 30 to 40 litres of fermenting must (sterilised) to 1,000 litres of wine sugared to promote bottle fermentation.

If a regular supply of absolutely pure yeast must be kept in stock, it is necessary to use the pure propagating apparatus designed by Hansen and by A Kuhle.

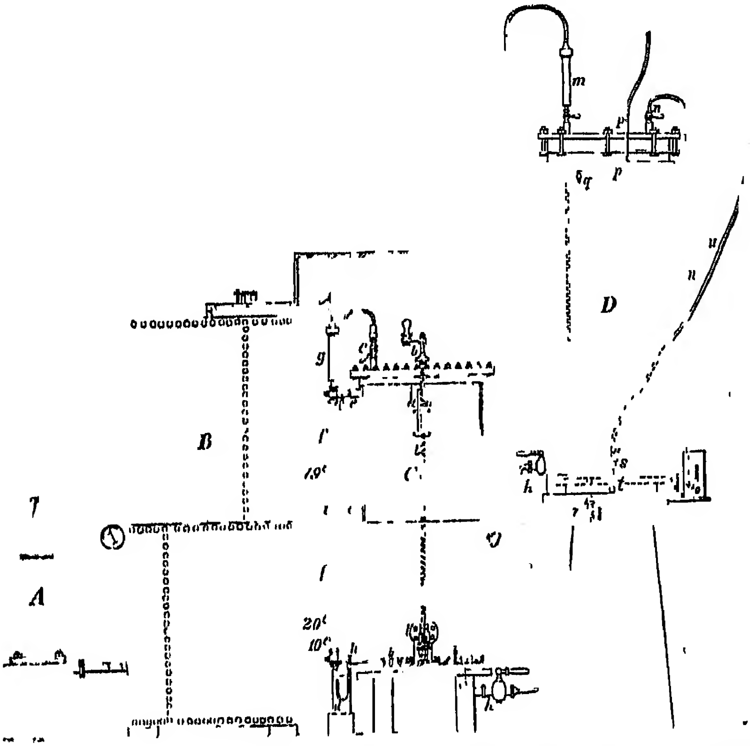

Fig. 98 Yeast propagating apparatus devised by Hansen and Kuhle —*A*, *air-pump*; *B*, *air-vessel*; *C*, *fermenting-cylinder*, *a*, window, *b*, *b*, *b*, stirrer, *c*, *c*, doubly-bent tube; *d*, vessel containing water, *l*, outlet cock; *f*, *f*, glass tube connected at *e* and *h* with the cylinder, and graduated for the measurement of fixed quantities of liquid; *g*, filter; *i*, rubber connection for glass tubes; *j*, tube with rubber connection for introducing the pure culture, *k*, *k*, connection with the *wort-cylinder* *D*; *m*, filter, *n*, *n*, doubly-bent tube; *o*, vessel containing water, *p*, *p*, spraying tube; *u*, connection with cook *s*, *t*, waste for cooling water.

The apparatus (Fig 98) consists of three chief parts and the necessary connecting tubes. First, the air apparatus, with air

pump (A) and air holder (B); secondly, the fermenting-cylinder (C); and, thirdly, the wort-cylinder (D) The air, which has previously been partially purified, is pumped into the receiver, and thence may be passed into either the wort or the fermenting cylinder In either case the air is sterilised by means of a cotton-wool filter (*g*, *m*) The wort cylinder is directly connected with the copper from which the boiling hopped wort is run in, it is then aerated in the closed cylinder, and is cooled by spraying.

The wort is then forced into the fermenting-cylinder, which, like the wort-cylinder, is constructed on the same principle as the ordinary two-necked flask. It is fitted with a doubly-bent tube (*c*, *d*), which dips into a vessel containing water, a vertical glass tube (*f*, *i*, *f*) for measuring the height of the liquid in the cylinder, an appliance (*b*, *b*) for stirring up the deposited yeast, and a specially constructed cock (*l*) for drawing off the beer and the yeast At about the middle of the cylinder there is a small side tube (*j*), fitted with india-rubber connection, pinch-cock, and glass-stopper. When a portion of the wort has been forced into the fermenting-vessel, the absolutely pure yeast —which is forwarded to the brewery in a flask specially constructed for this purpose—is introduced through the rubber tube at *j*; this is again closed, and the remainder of the wort may then be added either at once or after the lapse of a few days, according to the quantity of yeast introduced

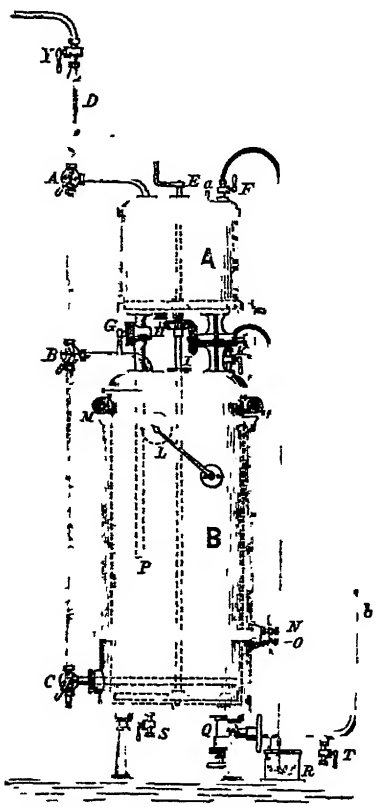

Fig. 99.—Yeast-propagating apparatus devised by Bergh and Jorgensen

Where it is necessary to regulate the temperature during fermentation, the fermenting-vessel is surrounded by a water-jacket.

By means of this simple apparatus it is possible to obtain, at short intervals, absolutely pure pitching yeast, sufficient for about eight hectolitres of wort, and when once started the apparatus works continuously.

Another type of propagating apparatus has been described by

Bergh and Jorgensen (Fig. 99). The filtered air passes through the three-way cocks at A, B, and C, into the two cylinders **A** and **B**. The upper cylinder holds about 50, the lower cylinder 160 litres. **A** is provided with a stirrer E, and a tube (a) for introducing the yeast and withdrawing samples. The bent tube F is an outlet for carbon dioxide. The tube $G\ P$ connects the two cylinders, and the connection can be made or broken by means of the cock G. H is the outlet for water used in cleaning A.

The cylinder **B** is surrounded by a cast-iron jacket made in two parts; the upper portion serves as a water-jacket for cooling the wort and for regulating the fermentation; the lower portion is used as a steam-jacket, and is provided with a cock at O as an inlet for the steam, and another at S as an outlet. M is a ring-shaped tube provided with small holes; this is connected with the cold-water main during the cooling of the wort; the water is drawn off at N. The stirrer J is set in motion by means of toothed gear. The height of the liquid in the cylinder is indicated by means of a float, with pointer and arc L. A bent tube, K, projects from the top of the cylinder. At the bottom is the cock O, which is connected with the pipe b by cock T. Both the bent tubes dip into the vessel R, which is filled with water.

The wort is introduced into the lower cylinder, where it is treated in the ordinary manner. The pure culture is introduced into the upper cylinder, and is then washed down into the lower cylinder by means of a little wort, which is forced from **B** into **A**, and then back again into **B**. When a vigorous multiplication of the yeast has set in, the liquid is stirred up, and a portion forced into **A**; this is to be used to start the next fermentation. The cylinder **B** thus serves alternately as fermenting- and wort-cylinder.

A comprehensive introduction to the method of dealing with the apparatus used in the laboratory for the preparation of pure cultures (moist chambers and flasks) is to be found, along with the mode of operating the two types of propagating apparatus, in a small hand-book of the author's, entitled *Practical Management of Pure Yeast*, London, 1903. Modifications of both forms of propagating apparatus have been described by Brown and Morris, Elion, Thausing, Van Laer, Pohl and Bauer, Wichmann, Fernbach, Jacquemin, and others. P. Lindner and Marx have constructed a somewhat different apparatus.

Seeing that in these machines the culture usually undergoes a large number of fermentations and under quite special conditions, it follows that some at least of the yeast races after leaving the plant may be found to have undergone some alteration when compared with the cultures described, which were grown in two or three small open vessels. Yet, on account of the great faculty for adaptation possessed by the yeast, it will usually behave quite normally when it has been propagated sufficiently to set up fermentation in

the first large tun If, after having completed the fermentation, the yeast is left for a considerable length of time in the cylinder, it will hardly be able to work normally until it has gone through a number of fermentations Top-yeast propagated in the cylinder is sometimes found to give rise to clarification trouble This is especially true of quickly clarifying races, and may be avoided by treating the original culture in such a way as to make it produce a normal clarification under all conditions An *exposé* of a special procedure with this object in view will be published later, when sufficient materials have been gathered, it will deal with the many different top- and bottom-fermentation types, and clear up the question completely

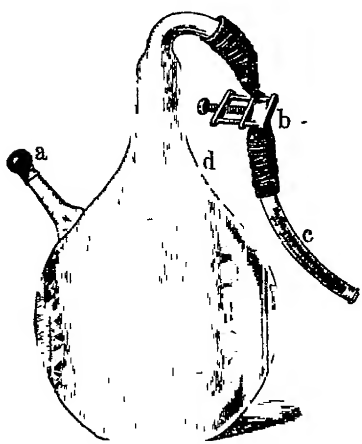

Fig 100

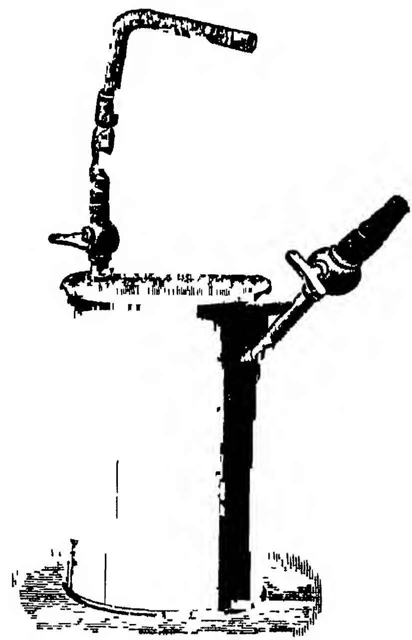

Fig 101

To facilitate sending the selected pure cultures in a liquid condition to a distance, special forms of flasks were devised by Hansen (Fig 100) and by the author (Fig 101) The yeast can be sent a great distance in these flasks, and there is no difficulty in safely transferring it from the flask to the fermenting-cylinder of the propagating apparatus, or to a small vat for development

In sending small quantities of pure cultures, in such a manner that they may be safely and readily employed for further cultivation, the small Hansen flasks are employed (p 33) They are connected, in the flame, with the Pasteur flask in which the pure culture has developed A trace of the yeast is transferred to the

cotton-wool, and the flask is again closed in the flame with the asbestos stopper, which is then coated with sealing-wax When the culture is to be used, the flask is again connected with a Pasteur flask containing wort, and the yeast is rinsed into the latter

This process has made it possible to send large collections of pure cultures to the most distant countries at a very small cost. Large cultures also, for direct use, can be despatched in a dried state to any distance

It is of the greatest importance to note that, even after the lapse of years, the particular yeast once selected can always be procured again, a sample of the pure culture being preserved in the laboratory in a 10 per cent solution of cane-sugar, kept in the flasks described in Chap i, devised by the author for the purpose It is of importance, in order that the culture yeasts may be kept unaltered for a long time, that the liquid should not evaporate, and that the layer of yeast deposited upon the bottom of the flask should not be frequently shaken (see Figs 7-9). During the introduction of a few drops into a Pasteur flask shaking can only be avoided by the use of the flask depicted in Fig 9 With any other variety it is necessary to maintain a number of flasks for each species of yeast, and each one will only serve for a few infections On the other hand, no effect of temperature has been observed during storage All physiological laboratories concerned with fermentation possess such collections of preserved growths. The author's collection of species which have been gradually introduced into practice dates back to the year 1884, and numbers many specimens Not a few of these species have retained those properties which are of industrial value for more than ten years. In the case of top-fermentation yeasts generally used in the *brewing* industry, it has, however, been found necessary, at not too long intervals, to renew the saccharose-culture by repeated fermentations in wort, in order to prevent them from losing the characters appreciated in practice According to Hansen's and the author's experiments yeasts may be kept alive under such conditions for many years

The *drying* of *industrial yeast* for preservation and despatch has been carried out by different methods from time to time. Extensive experiments were undertaken by Will with mixtures of washed and pressed brewery bottom-yeast with infusorial earth, gypsum, charcoal, asbestos, etc , the mixture, after drying at a temperature rising to 40° C, was placed in tins, which were soldered Even 13 years after the yeast was submitted to this treatment, living culture-yeast cells could be found in a charcoal mixture, and after 17 years wild yeast cells were still alive in an asbestos mixture The best preservatives were found to be charcoal and gypsum, acting as dehydrating agents Heron used a mixture of yeast and dextrose, kneaded together and solidified One special

treatment consists in liquefying pressed yeast by an addition of sugar (10 per cent) and then drying the liquid yeast at 50° C Brewery yeast must first be deprived of part of its albuminoid matter by aeration in the sugar solution Yeast treated in this way is said to retain some 90 per cent of its cells alive. By another method yeast is grown for some hours in a very dilute sugar-solution, and mixed with a small dose of asparagine (5 g per 50 kg) before pressing and drying Direct drying of industrial yeast without employing any admixture, is accomplished by various processes, in which the pressed yeast, either in thin layers or in the form of threads or rods, is exposed either to the usual or to a rising temperature, the moisture being quickly removed by a rapid current of air It has proved greatly beneficial to expose the yeast before drying to intense aeration in an aqueous or a dilute sugar solution or a very dilute malt extract The aeration is stopped when the cell-vacuoles have disappeared, the yeast is then centrifuged and dried at 55° to 65° C until the amount of water is reduced to 10 per cent The dried yeast is put with a hygroscopic substance into air-tight canisters

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2249

INDEX.